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Reverse engineering of short-chain fatty acid tolerance and production in Escherichia coli

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Reverse engineering of short-chain fatty acid tolerance and production in *Escherichia coli*

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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TABLE OF CONTENTS

Page

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ABSTRACT

Product toxicity is a common problem in microbial production of biorenewable fuels and chemicals. Historically, the field of metabolic engineering has relied on enrichment of expression libraries and strain evolution for improving tolerance. Reverse engineering of these strains can aid in the identification and development of rational design strategies for strain improvement, according to Orgel's Second Rule that "evolution is cleverer than you are".

Here, we investigated the evolved strain with increased short-chain fatty acid tolerance and 5-fold higher fatty acid titer relative to its parent strain to discover and understand the mechanisms of the phenotypic changes. Four mutations were identified in the evolved strains, as well as the chronological order of mutations during adaptive evolution. Then we studied each mutation and their synergistic interaction by characterizing the reconstructed strains, which had single mutation, double mutation or triple mutations replaced into the genome of the parent strain. The *waaG* mutation contributed to increased C8 tolerance, fatty acid titer, and membrane integrity. The increased fatty acid titer was mainly affected by the mutant *rpoC* gene. The decreased membrane fluidity and increased cell surface hydrophobicity of evolved strain were caused by the synergistic interaction of *waaG*, *rpoC*, and *basR* mutations. We also noticed each mutation was able to alter the membrane lipid composition differently. The association between mutations and phenotypic changes we identified could be used as rational engineering strategies for improving tolerance and production of microbial biocatalyst.

However, the *rpoC* and *basR* encode transcriptional regulators, and we were not able to fully understand the mechanisms of these two mutations by only genome-level reverse engineering. Thus, we further investigated these two mutations by transcriptome analysis. Compared evolved strain LAR1 to parent strain ML115 during fatty acid producing, twenty-nine

genes made statistically significant changes in transcript abundance, which could be influenced by *rpoC* mutation. Also, nine genes were identified had differential transcript abundance, which could be impacted by the *basR* mutation. Characterization of these interesting genes is in progress to discover the mechanisms of increased short-chain fatty acids tolerance and production of evolved strain LAR1.

In order to deepen our understanding the association between fatty acid production and cell membrane properties, we characterized a small group of environmental *E. coli* isolates with significantly higher short-chain fatty acids production in minimal medium relative to lab strain MG1655. Consistent with previous studies, decreased membrane fluidity was associated with increased fatty acid production. The C16:1/C16:0 ratio (mol/mol) was suggested to be one of the important metrics, when rationally engineering membrane lipid composition for improving short-chain fatty acids production in *E. coli.* We also discovered the direct association between cell surface hydrophobicity and short-chain-fatty acids titer. In short, the findings of our work could provide new insights into the mechanisms of short-chain fatty acid tolerance and production, and rational engineering strategies for improving performance of biocatalyst.

CHAPTER 1. INTRODUCTION

Background

Increasing attention has been paid to engineering microorganisms to produce biofuels and biorenewable chemicals using sustainable carbon-based sources in order to mitigate environmental pollution of producing petroleum-based fuels and chemicals and to meet the increasing global demand for energy (1–4). In this work, we focus on one of the attractive biorenewable chemicals, fatty acids, which have a large and increasing market due to their wide range of applications as a multifunctional precursors (5–12). However, it is a common problem that the desired products are toxic to the engineered microbial biocatalyst, which appears to impair cell growth and limit fermentation performance (13–18). Extensive work has been done to characterize the inhibition of ethanol and butanol in *Escherichia coli* and yeast (19–22). However, more efforts were needed in order to understand the mechanisms of the fatty acid toxicity in microbial biocatalysts.

Biorenewable processes such as the production of ethanol, glycerol, 1,3-propanediol, and lactic acid have already been industrialized (3). Many reports have been published on increasing fatty acid production in engineered *E. coli* strains or yeast (23–27) while the inhibition of short-chain fatty acids has also been observed (23, 27). Thus, it is possible to industrialize the production of short-chain fatty acids, if the titer, production, and yield of engineered biocatalyst could achieve the commercial level. In order to meet this objective, we need to eliminate or at least largely alleviate the inhibition of short-chain fatty acids. The first important step is to characterize and understand the mechanisms of the inhibition. Transcriptome analysis and cell characterization study both proved that short-chain fatty acids damage the cell membrane during fatty acid production in *E. coli*, which seems to be the major mechanism of toxicity (27, 28). In

addition, it has also been reported that the membrane damage and intracellular acidification are caused by exogenous challenge with octanoic acid (C8) at pH 7.0 in *E. coli* (14, 29). In the past few years, many efforts have been made to address the membrane damage issue in order to increase the short-chain fatty acid tolerance and production in *E. coli*. Altering membrane lipid composition successfully increased the membrane integrity and fatty acid tolerance, while it did not increase fatty acid productivity (28). Preventing medium-chain fatty acid incorporation into the cell membrane successfully increased the fatty acid tolerance and production (30). Synthesizing trans unsaturated fatty acids and incorporating them into the cell membrane improved *E. coli* robustness and fatty acids production (31).

In our previous work, we applied adaptive laboratory evolution to *E. coli* to improve exogenous octanoic acid tolerance. The evolved strain had a 5-fold increase in fatty acid production, increased membrane integrity, and increased tolerance to not only short-chain fatty acids but also butanol isomers (23). It is important to discover and understand the mechanisms of the phenotypic changes of the evolved strain by reverse engineering, which can be used as design strategies of rational engineering for biocatalyst improvement. Reverse engineering is a powerful tool, which contributed to improving succinate production in *E. coli* (32), increasing yeast membrane thermotolerance (33), and identification of the major furfural reductase in *E. coli* (34).

In this work, we investigated the evolved strains by whole-genome sequencing, transcriptome analysis and strains characterization, in order to discover the mechanisms of increased short-chain fatty acid tolerance and production, and altered cell membrane properties. Four mutations were identified in the evolved strains, and the association between mutations and phenotypic changes were discovered by us. Two mutations encode transcriptional regulators, thus we did further investigation by transcriptome analysis. Characterization of the genes with

differential transcript abundance is in progress to have a better understanding of the evolved strain. At the same time, cell membrane properties of a small group of environmental *E. coli* isolates with high short-chain fatty acids production were investigated. We determined that cell membrane fluidity and cell surface hydrophobicity were associated with short-chain fatty acid titer in *E. coli*. Overall, our work focuses on discovering and understanding the mechanisms of increased short-chain fatty acids tolerance, high fatty acid production, and altered cell membrane characterizations. These mechanisms could be used as rational engineering strategies for improving performance of engineered microbial biocatalyst.

Thesis Organization

The thesis is composed of seven chapters. They are organized as follows: Chapter 1 is a general introduction of background and the organization of this thesis. Chapter 2 is a book chapter that reviewed the evolutionary methods for improving production of biorenewable fuels and chemicals. Chapter 3-6 are papers either published, under review or intended for submission for publication regarding the topic of increasing short-chain fatty acids tolerance and production in *E. coli.* Chapter 7 is a summary of the all work as well as insights into future work.

Chapter 2: Evolutionary Methods for Improving the Production of Biorenewable Fuels and Chemicals

Chapter 2 is a book chapter about how evolution works, the use of evolution to improve the tolerance and production of biorenewable fuels and chemicals, and the application of reverse engineering to identify the inhibition mechanisms of inhibition compounds.

Chapter 3: Evolution for Exogenous Octanoic Acid Tolerance Improves Carboxylic Acid Production and Membrane Integrity

Chapter 3 is a research paper overall introducing the evolved strains which are the important research subjects of my studies, including how we obtained the evolved strains, and phenotypic changes of evolved strains. My contributions to this work include assessing cell membrane integrity, isobutanol and *n*-butanol tolerance, and preparing the manuscript.

Chapter 4: Genome-level Reverse Engineering of *Escherichia coli* Evolved for Increased Short-chain Fatty Acid Tolerance and Production

Chapter 4 is a research draft about investigating the evolved strain to discover and understand the mechanisms of the phenotypic changes by genome-level reverse engineering. We identified four mutations in evolved strains, and explored how each mutation and their synergistic interaction contributed on increased short-chain fatty acid tolerance, improved fatty acid titer, and altered cell membrane characterizations.

Chapter 5: Transcriptome analysis of *Escherichia coli* evolved for Short-chain Fatty Acid

This chapter is a research draft about further studying the evolved strain by transcriptome analysis to discover the mechanisms of the two mutations which encode transcriptional regulators. Characterization of the genes with differential transcript abundance between evolved strain and parent strain is in progress.

Chapter 6: Lessons in Membrane Engineering for Octanoic Acid Production from Environmental *Escherichia coli* Isolates

Chapter 6 is a submitted manuscript about characterizing the cell membrane of a small group of environmental *E. coli* isolates with high short-chain fatty acid production, in order to learn design strategies of membrane engineering to improve short-chain fatty acid production.

Chapter 7: Summary and Future Work

The final chapter summarizes the important findings in this work, proposes future work that can lead to deepen understanding of this research topic.

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CHAPTER 2. EVOLUTIONARY METHODS FOR IMPROVING PRODUCTION OF BIORENEWABLE FUELS AND CHEMICALS

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Abstract

Evolutionary methods to improve strain performance are a powerful complement to rational, predictive strain engineering. These evolutionary methods can consist of serial transfers or engineered methods, such as global transcription machinery engineering (gTME) and the SCALEs expression libraries. The types of improvements achieved in strain performance include increasing the tolerance to inhibitory products, such as alcohols and carboxylic acids, and increasing the tolerance and utilization of biomass-derived sugars, such as pentose sugars and biomass hydrolysate. In some cases, the selective pressure applied during the evolutionary process has enabled co-selection of growth and strain performance. This approach has proven successful for the production of ethanol, lactic acid, succinate and alanine. In other cases, strains are evolved for tolerance to exogenously-supplied inhibitors, with the intention of increasing production by increasing tolerance. This has been successful in some cases, particularly in regards to ethanol production. Identification of the mutations that enable the evolved phenotype and understanding the molecular basis of their contribution to strain fitness and performance can reveal novel strategies for increasing tolerance. In this manner, our knowledge of possible design schemes and our understanding of our workhorse biocatalysts are increased. Reverse engineering has provided rational design strategies for increasing tolerance to isobutanol, furfural and ethanol as well as increasing the production of succinate.

Introduction

Metabolic engineering is, by definition, a rational and predictive field [1,2]. This type of deliberate, planned approach has enabled the production of a wide variety of biorenewable fuels and chemicals, including but not limited to ethanol, butanol, styrene and carboxylic acids [3-6]. Similarly, an engineering approach has proven useful in enabling the utilization of renewable biomass-derived carbon sources, such as pentose sugars [7-9], anhydrosugars [10], and syngas [11,12]. However, our incomplete and imperfect knowledge of microbial metabolism, physiology and regulatory networks hinders our ability to consistently and efficiently obtain strains with sufficiently high titer, yield and/or productivity through rational engineering alone.

One of the challenges frequently associated with production of biorenewable compounds at a high yield and titer from biomass-derived substrates is biocatalyst inhibition [13-19]. This inhibition can either be from the desired product or from non-sugar components of the “dirty” feedstock. This inhibition can sometimes be mitigated via *in situ* product removal [20-25] or treatment of the feedstock [26-35], though this may incur an unacceptable increase in cost.

Another option for dealing with this inhibition is to modify the biocatalyst for increased tolerance of the inhibitory compounds. Metabolic engineering for increased tolerance has included strategies such as provision of the appropriate exporter [36,37] and engineering of stronger membranes [38,39]. However, this rational approach to increasing tolerance can be limited when either the mechanism of toxicity is not known and/or there is not a clear engineering strategy to mitigate the identified inhibition mechanism. Omics analysis can prove quite useful in identifying the mechanism of inhibition [40-43], though interpretation of omics data is dependent upon the quality and thoroughness of enzyme annotation and regulatory network characterization.

Evolutionary strategies for improving biocatalyst performance provide an excellent counterpoint to the rational design strategy. Evolution can be used to improve tolerance to exogenous inhibitors, improve utilization of a target substrate or improve production of the target compound. Such strategies are not hindered by erroneous or incomplete enzyme annotation or lack of regulatory network characterization. When the appropriate selective pressure is applied and the key mutations supporting the evolved phenotype are identified, novel design strategies can be distilled and our understanding of the biocatalyst can increase. As we increase the pool of available design strategies and our understanding of the organism, our ability to apply rational, predictive engineering strategies also increases.

The goal of this chapter is to describe the use of evolution to improve production of biorenewable fuels and chemicals. Other similar works have been published recently on this topic [44-53]. Here we given an overview of how evolution works, the development of evolution-based strain design tools, evolution to increase tolerance to exogenously supplied inhibitors, evolution to improve strain performance, and reverse engineering to identify design principles. Note that evolutionary efforts that target specific enzymes have recently been reviewed elsewhere [54-56] and are not discussed here.

Mechanisms of Evolution

The power of natural selection is promising and very useful for the modification of microorganisms for a variety of properties. Put more succinctly as Orgel's 2nd rule, "evolution is cleverer than you are" [57,58]. In this work, we are interested in the use of adaptive evolution to improve microbial production of biorenewable fuels and chemicals, such as biocatalyst tolerance to toxic compounds or operating conditions, or to otherwise improve strain performance.

How does evolution work?

The essence of evolution is perturbation of the DNA sequence. These changes can occur within coding regions (genes) or non-coding regions. Changes within coding regions can either impact the resulting amino acid sequence, or be “silent” mutations that change the codon identity but not the resulting amino acid. Changes to the genome sequence can occur through a variety of events, as described elsewhere [59-61]. These events can involve changes of single nucleotides or insertion or deletion of various-sized segments.

Single base-pair (point) mutations can arise either through damage to the DNA or during DNA replication. The physical structure of the nucleotides can be impacted by mutagens, such as radiation or ethidium bromide [62,63]. Mutations can also arise via incorporation of the wrong nucleotide during DNA replication though proofreading systems exist to detect and repair these types of errors, they are not infallible [64,65].

As opposed to the single base-pair changes described above, many observed mutations involve the insertion or deletion of more than just one base pair. These types of insertions and deletions can possibly arise via transposons and retrotransposons. Transposons are a class of genetic elements which can duplicate themselves and move to different position within a genome. Retrotransposons are a subclass of transposons, which need RNA to be intermediates. Transposons can have dual impacts on their host, in that they can confer mutations that are either beneficial or harmful. However, in adaptive evolution, transposons are considered to be an accelerator of the evolutionary process [66]. Large-scale genomic rearrangements have also been observed [67]. Another possible mutation type is the insertion or deletion of a small number of nucleotides, i.e. in-frame deletion of a single amino acid [68].

Most microorganisms have a relatively low and stable mutation rate. For example, *E. coli* averages 0.0025 mutations per genome per replication and *S. cerevisiae* maintains 0.0027 mutations per generation [69]. Mutations to the proofreading systems, general stress response or DNA repair systems can increase this mutation rate [60,61,70]. For example, RpoS is a well-characterized regulator of the general stress response and two mechanisms of the RpoS-mediated response can increase the mutation rate of stressed cells. One mechanism is the increased expression of the error-prone DNA polymerase, Pol IV; the other is the decreased expression of the mismatch repair enzymes [60,61,71].

Thus, there are a variety of natural mechanisms for generating diversity in microbial genomes (Table 1). These mechanisms include single base pair changes, either within a coding or non-coding region; short insertions or deletions that can possibly truncate a gene or change the number of amino acids; gene silencing or increased expression via transposon insertion; or large-scale rearrangements in genome organization. One can envision that these changes can influence the strain behavior in a variety of ways, including but not limited to perturbation of transcript abundance, transcript stability, translation initiation, translation efficiency and enzyme amino acid sequence. This change in amino acid sequence could impact protein function, protein stability and interaction with other cellular components. In this manner, directed evolution enables screening of these variants and selection of those that confer the desired behavior.

Directed evolution

Directed evolution performed in the laboratory seeks to exploit the natural evolution process in order to obtain strains with desired properties. There are a variety of techniques for guided laboratory evolution. Each of these essentially aims to obtain evolved strain(s) by

propagating the parent strain in a controlled environment. The specifics of the propagation methods and the controlled environment depend on the experimental purpose.

A traditional example of this propagation is the sequential transfer method [72-74]. Essentially, cells are grown in a condition which is stressful enough to slow growth, but not stressful enough to halt DNA replication. As the cells grow in this stressful condition, some will acquire mutations, as described above. Those that have acquired a mutation that enhances growth in the condition of interest will grow faster than their cohorts. As additional beneficial mutations are acquired, one strain will eventually become the dominant strain within the population. During this time, the selective pressure can be increased. Since mutations are largely acquired during the growth phase and since growth is the selective marker, it is desirable to maintain the cells in a state of near-continual growth. Ideally, such growth would involve the use of a carefully-maintained chemostat culture. However, in practice this is often approximated through the use of serial batch cultures. Since the culture at the end of the serial transfer process presumably contains a variety of strains, it is very important to isolate and characterize single colonies before proceeding. Briefly, the culture is diluted into new growth medium on a regular basis, i.e. every 24 hours, and the magnitude of the selective pressure is gradually increased. Finally the evolved strain is obtained when the cells display the desired behavior. Here we briefly describe several cases where this type of approach was used.

In order to increase thermal tolerance, *E. coli* cells were sequentially transferred in minimal medium for 523 days. During the course of this evolution, the temperature was increased from 36.9˚C to 44.8˚C. The final evolved strain was able to grow at 45.9˚C, while the original parent strain cannot grow at 43.2 ˚C [72].

A variety of studies have aimed to increase ethanol tolerance. For example, ethanologenic *E. coli* KC01 was sequentially transferred in rich medium with 50 g L^{-1} xylose for 6 weeks while the concentration of ethanol was increased from 10 g L^{-1} to 40 g L^{-1}. The evolved strain SZ470 could successfully grow anaerobically in the presence of 40 g L^{-1} ethanol. This represents a two-fold increase in ethanol tolerance relative to the parent strain [73]. Yomano *et al* took a slightly different approach to selecting for ethanol tolerance. Ethanologenic *E. coli* strain KO11 was subjected to alternating rounds of growth in liquid and on solid media. The liquid media contained selective concentrations of ethanol. At the end of the growth period on liquid media, cells were plated onto solid media and colonies with high ethanol production capability were identified based on size and morphology [75]. This alternation between liquid and solid media represents a useful deviation from the standard liquid-to-liquid serial transfer method.

Recently, a new sequential transfer method was created and demonstrated by increasing n-butanol tolerance in *E. coli*. This new method is named "visualizing evolution in real time" (VERT) [76,77] and is based on traditional evolution, but can track distinct improvements in fitness by fluorescence-based techniques [76,77]. In the VERT scheme, subpopulations of the parental strain are generated. These subpopulations are isogenic except for the recombinant expression of distinct fluorescence proteins. These fluorescence reporters enable rapid determination of the population distribution. When the various subpopulations are growing at similar rates, the population distribution is roughly equal between the various groups. When a cell in one subpopulation acquires a useful mutation that enables faster growth, this subpopulation will become dominant. This alerts researchers that a useful mutation has occurred.

Engineered Methods of Evolution

Modification of microbial biocatalysts for some complex functionalities, such as increased tolerance or production of desired compounds, can require perturbation of multiple genes. However, our ability to rationally plan and then introduce these modifications can be limited by our knowledge of microbial physiology and metabolism and resource availability. These combinations can also require unacceptably lengthy directed evolution schemes.

Global transcription machinery engineering (gTME)

Global transcription machinery engineering (gTME) aims to introduce these multiple perturbations to the transcriptome by altering the transcription machinery. This method has proven to be an appealing and efficient tool to improve the performance of biocatalysts [78].

One of the first examples of gTME was the improvement of ethanol tolerance and production by *S. cerevisiae* [79]. A library of mutated versions of the *SPT15* gene, which encodes the TATA-binding protein, was generated by error-prone PCR. This led to increased ethanol tolerance and yield, as described below. *SPT15* was also selected for mutation in a gTME project that aimed to improve xylose tolerance and consumption by *S. cerevisiae* [80]. The mutant library was screened in xylose defined medium; the resulting strain showed a conversion efficiency of 93.5% for 50 g L^{-1} xylose. This conversion efficiency is comparable to the 98.3% observed for 50 g L^{-1} glucose. Even when glucose and xylose were supplied as a ratio of 1:1, the utilization was 97.3% and 90.8% respectively.

The power of gTME has also been demonstrated in *E. coli* [78]. The *rpoD* gene, which encodes the main sigma factor σ^{70}, was subjected to random mutagenesis, along with its promoter region, for three rounds of error-prone PCR. After screening and selection from the library in the presence of 50 g L^{-1} ethanol, the best mutant showed better growth in the presence

of 20 – 70 g L^{-1} ethanol relative to the strain harboring only the wild-type *rpoD*. gTME was also demonstrated to increase the production of lycopene by 50% and increase tolerance to multiple inhibitors, such as sodium dodecyl sulfate.

Global transcription factor cyclic AMP receptor protein (CRP) of *E. coli* was subjected to gTME for increasing the tolerance to 1-butanol [81]. Four mutants with improved performance were selected from the library. DNA shuffling was then used to combine these beneficial mutations. The final mutant MT5 showed a growth rate of 0.18 h^{-1} in the presence of 9.7 g L^{-1} 1-butanol, which was twice as high as that observed for the parent strain.

Thus, gTME has proven to be an effective method for enabling complex phenotypes in a variety of organisms. These complex phenotypes include tolerance of inhibitory compounds and production of target compounds.

SCALEs

gTME aims to perturb the expression of a large number of genes by changing the sequence, and therefore behavior, of proteins involved in controlling gene expression. SCALEs aims to perturb the expression of multiple genes by increasing the copy number of genomic DNA fragments of various sizes. Libraries of cells containing these fragments are then enriched for their ability to confer a phenotype of interest. The cells isolated by this enrichment procedure are typically then subjected to a genome-wide, microarray-type multiscale analysis [82].

For example, *E. coli* K12 was used to construct five genomic libraries for the demonstration and validation of SCALEs [82]. Transformants carrying the plasmid -based DNA fragments of different sizes were cultured in a continuous fermentation system for selection and sampled periodically. Microarray and multiscale analysis was then conducted on the enriched plasmids to identify the position and size of the inserts contributing to the increased fitness.

This approach was used to identify *E. coli* K12 genes responsible for the increased tolerance of *E. coli* to acetate. This interest in acetate tolerance was motivated by the fact that acetate is a common inhibitor found in biomass hydrolysate [83]. The populations surviving in the presence of 1.75 g L^{-1} acetate were selected and subjected to microarray and multiscale analysis. In this manner, a list of genes whose overexpression contributed to high acetate tolerance was obtained. This list includes such genes as *murC* for peptidoglycan biosynthesis and *yjdL* for dipeptide transporter.

The SCALEs method was also employed to improve the growth of *E. coli* NZN111 [84]. Previous metabolic engineering of NZN111 had resulted in a growth deficiency due to a high $NADH/NAD^+$ ratio. The SCALEs analysis led to the identification of 9 *E. coli* K12 genes that can increase the growth of NZN111 approximately 5-fold when their expression was increased. The enriched population also showed a 20% increase in succinate production.

Other methods for improving the relative fitness of biocatalysts include COMPACTER [85] and TRMR [86].

Evolution for Increased Tolerance

As described in many recent reviews [17,87-89], the metabolic engineering of biocatalysts for economically-viable production of desired compounds is often restricted by product-mediated inhibition, such as that imposed by organic solvents [90,91] and carboxylic acids [18]. Additionally, low-cost, renewable feedstocks, such as those derived from biomass or industrial byproducts, often contain compounds that can inhibit biocatalyst growth and metabolism [92]. One approach for dealing with this inhibition is selective removal of the inhibitory compound as it is formed [93-95]. Another approach is to evolve the microbes for improved tolerance of the inhibitory compounds [19,96] (Table 2). Performing reverse

engineering to reproduce the phenotype or trait of interest can aid in the identification of design principles and increased characterization of the host microbe, as described below.

Organic solvents, including next-generation biofuels such as butanol [19], are highly toxic to microorganisms. This toxicity is largely attributed to their hydrophobicity and the resulting damage to the cell membrane [97,98]. A variety of rational engineering strategies have been applied to increase the tolerance of biocatalysts to these inhibitory products. Here we briefly summarize two of these. As mentioned above, provision of biocatalysts with appropriate efflux pumps has been shown to increase tolerance to inhibitory products [19]. For example, *marA*, which is responsible for activation of the AcrAB-TolC efflux pump, was overexpressed in *E. coli* leading to the increased tolerance to geraniol [99]. On solid media with 1.0% (v/v) geraniol, the strain harboring overexpressed *marA* gene showed 10^4-fold colony forming efficiency compared to that of the parent strain. Additionally, the intracellular accumulation of geraniol was only half of the parent strain. Organic solvents can interfere with appropriate protein folding, and thus the GroESL chaperone system, which facilitates protein folding, was overexpressed in *E. coli*. The resulting strain had increased tolerance to organic solvents such as ethanol, *n*-, 2-, *i*-butanol and 1, 2, 4-butanetriol [100].

The standard sequential transfer method has been used in a variety of cases to adapt microbes to inhibitory environments (Table 2) [101]. Here we summarize a few of these instances. Uses of modified evolution techniques are described at the end of this section.

Directed evolution was conducted in batch fermentors to acquire a strain of *E. coli* with increased isobutanol tolerance. After 45 sequential transfers, tolerance increased from 4 g L^{-1} to 8 g L^{-1} [102]. This demonstrates the ability of the sequential transfer method to obtain significant gains in tolerance in a relatively short time. In another isobutanol-focused project, parallel

directed evolution was applied to multiple populations of *E. coli* simultaneously in minimal medium containing isobutanol with either glucose or xylose as carbon source [103]. After approximately 500 generations in glucose medium and 430 generations in xylose medium, the tolerance of the mutants was improved from 0.75% (w/v) to 2% (w/v) and 1.75% (w/v), respectively. However, the strain evolved on glucose also showed increased isobutanol tolerance in xylose minimal media and the strain evolved on xylose showed increased isobutanol tolerance in glucose minimal media. Thus, the specificity of the adaptation in the two carbon sources was relatively low. When these evolved strains were further characterized in rich media with isobutanol, there was no significant gain in fitness relative to the parent. These results highlight the fact that selected mutations can be specific to the experimental conditions (media type) while other aspects of the experimental condition can be less impactful (carbon source).

Another evolutionary approach to improve isobutanol production by *E. coli* focused on tolerance of the valine analog norvaline [104]. It should be noted that the isobutanol production pathway in this case is this same pathway that is used to produce valine. Through a combination of mutagenic treatment, selection for growth in the presence of norvaline, and repair of a mutation within *rpoS*, the final strain NV3r1 was obtained. This strain produced 21.2 g L^{-1} isobutanol at a yield of 0.31 g g^{-1}, relative to the 12.0 g L^{-1} and 0.24 g g^{-1} observed for the parent strain.

Directed evolution has also been applied to microorganisms other than *E. coli*. A butanol-tolerant *S. cerevisiae* strain was obtained after 30 serial transfers in the presence of 2-butanol [105]. The resulting mutant was able to maintain growth in 3% (v/v) 2-butanol, a concentration which completely inhibited growth of the parent strain. The evolved strain also showed increased tolerance to other butanol isomers. Specifically, the evolved strain was able to grow in the

presence of 1.9% (v/v) 1-butanol and 1.9% (v/v) iso-butanol, while no growth was observed for the parent strain in these concentrations. Similarly, when *Pseudomonas putida* was evolved for cyclohexane tolerance, it also acquired enhanced tolerance to the organic solvents decalin, methyl cyclohexane and toluene [106]. This evolved strain was acquired by only 12 sequential transfers with cyclohexane content increased from an original value of 5% (v/v) to a final concentration of 60% (v/v). Note that evolved strain was evaluated in 60% (v/v) of each of the solvents listed. These two studies demonstrate that adaptive mutations that confer tolerance one inhibitor can also be beneficial in conferring tolerance to other, chemically similar, inhibitors.

Carboxylic acids are very appealing biorenewable chemicals for synthesizing a variety of commodity chemicals [107,108]. However, as with organic solvents, the fact that they hinder the performance of the biocatalyst is still an issue that needs to be addressed for economically viable production [109,110]. Many rational engineering efforts have been taken to improve the resistance of microbes to the free fatty acids. For example, the Aas pathway for incorporating medium-chain fatty acids into membrane phospholipids was deleted in *E. coli* with the expression of the gene encoding cytosolic acyl-ACP thioesterase from *Umbellularia californica* to alleviate the toxicity and restore the strength of cell membrane [111]. The CFUs (colony-forming unit) of the engineered strain were twice as high as those of the parent strain in the presence of 1 g L^{-1} of 12:0 and 14:0 fatty acids. By employing an alternative thioesterase from *Geobacillus sp.*Y412MC10, the membrane content of unsaturated fatty acids was decreased, thus increasing the tolerance to the free fatty acids [38]. An evolutionary approach has also been used to improve tolerance to octanoic acid in *E. coli* [112]. Specifically, 17 transfers were performed in glucose minimal media over the course of approximately 700 hours. Initially, 10 mM octanoic acid was used, though the concentration was gradually increased to 30 mM. The resulting

evolved strain showed increased tolerance not only to octanoic acid, but also to hexanoic acid, decanoic acid, and somewhat surprisingly, isobutanol and n-butanol.

In addition to dealing with inhibitory products, biocatalysts can also be limited by the inhibitory compounds contained in the substrates. Biomass-derived sugars have been utilized as substrate for the production of biorenewable chemicals and fuels [113,114]. However, inhibitory compounds are often formed during the pretreatment process [16,115]. Some of these compounds, including furan derivatives, aldehydes and organic acids, have been under investigation regarding their impact on microbes [16,43,116-118]. Furfural is the most thoroughly characterized of these biomass-associated inhibitors [119-125].

A furfural-resistant *E. coli* strain EMFR9 was derived from ethanologenic LY180 based on growth in batch fermentors with gradually increased furfural concentration from 0.5 g L^{-1} to 1.3 g L^{-1} during 54 serial transfers [122]. In minimal medium with 100g L^{-1} xylose, this resulting strain could produce approximately 40 g L^{-1} ethanol in the presence of 1.0 g L^{-1} furfural; the parent LY180 could not even maintain growth in such conditions. The increased tolerance of EMFR9 to furfural also resulted in increased tolerance to 5-hydroxymethyl furfural (5-HMF) [126]. Specifically, EMFR9 produced more than 40 g L^{-1} ethanol after 96 hr in minimal medium with 100 g L^{-1} xylose and 2.5 g L^{-1} 5-HMF. Note that 1.0 g L^{-1} 5-HMF completely inhibited growth of the parent strain.

Directed evolution has also been applied to directly improve the tolerance of biocatalysts to lignocellulosic biomass hydrolysate. *S. cerevisiae* strain TMB3400 was evolved in batch cultures with 20 g L^{-1} glucose and xylose respectively and 12 inhibitory compounds. Specifically, these cultures included HMF, furfural, acetic acid, formic acid, levulinic acid, vanillin, syringaldehyde, coniferyl aldehyde, hydroquinone, cinnamic acid, 4-hydroxybenzoic

acid and guaiacyl acetone, each of which have been detected in spruce hydrolysate [127]. When characterized in the presence of 20 wt% of this simulated spruce hydrolysate “cocktail”, the evolved strain’s maximum specific growth rate (0.33 h^{-1}) was double that of the parent and the length of the lag phase was similarly reduced 2-fold. The original parent strain was also evolved in a chemostat for 97 generations using actual spruce hydrolysate, as opposed to the simulated cocktail. Initially, 20% (v/v) hydrolysate was used and the concentration was gradually increased to 50% (v/v). Mutants were selected from both evolutionary procedures and further characterized. Strains from both groups demonstrated a 25 – 38% increase in specific sugar consumption rates and a 32 – 50% increase of specific ethanol productivity compared to the parent strain using actual spruce hydrolysate.

Modified evolutionary strategies have also been used to increase tolerance to organic solvents. gTME was used on the global transcription factor cAMP receptor protein (CRP) for improving the tolerance of *E. coli* to isobutanol [128]. One of the resulting mutants showed growth of 0.18 h^{-1}, compared to 0.05 h^{-1} of the parent strain, in 9.6 g L^{-1} of isobutanol.

VERT was applied to *E. coli* BW25113 for improving its tolerance to *n*-butanol in chemostat with M9 minimal medium and 5 g L^{-1} glucose [129]. After 144 generations, the resistance of the evolved strain was increased from 0.5% (v/v) to 1.3% (v/v).VERT was also used to identify the adaptive events of *S. cerevisiae* derived from FY2 during corn stover hydrolysate utilization [130]. The growth rate of the best mutant was increased by 57% in 25% (v/v) hydrolysate relative to the parent strain.

Here we have described the use of serial transfer-based evolution, gTME and VERT to increase the tolerance of a microbial biocatalyst to specific inhibitory compounds. It can be seen that these are effective tools and sometimes the acquired tolerance also applies to other

compounds. However, as described in the next section, acquiring tolerance with the goal of increasing production of the focal compound, has proven challenging.

Evolution for Increased Production

Besides enhancing biocatalyst tolerance to both substrates and products, metabolic engineering of biocatalysts for improving their performance of production is also of great importance. In some cases, evolution-based strategies can be designed so that selecting for cells with faster growth co-selects for strains with improved production abilities (Table 3).

E. coli W was engineered for ethanol production by chromosomal insertion of the *pdc* and *adhB* genes from *Zymomonas mobilis* [131]. Successive dilutions were transferred to solid media for selecting large colonies with higher expression of these two genes, enabling higher ethanol production. Serial transfers in rich media with 10% glucose were then used to stabilize both the alcohol dehydrogenase activity and the high resistance to Cm, where Cm resistance is indicative of *pdc*/*adhB* expression. These serial transfers were performed for more than 60 generations. The resulting strains were able to produce more than 50 g L^{-1} ethanol from 100 g L^{-1} glucose and more than 40 g L^{-1} ethanol from 80 g L^{-1} xylose.

E. coli strain TCS009, a derivative of MG1655 engineered for production of ethanol from glycerol, was also improved through co-selection of growth and ethanol production [132]. After 500 serial dilutions in minimal media with glycerol as sole carbon source, the specific growth rate had increased 1.8-fold. Ethanol production by this evolved strain was also improved both in terms of yield and rate. Specifically, the evolved strain produced ethanol from glycerol at a yield of 0.45 ± 0.01 g/g^{-1} over 48 hours, while its parent had a yield of 0.34 ± 0.00 g/g^{-1} over 72 hours.

This growth-based strain improvement was also used as part of the engineering of *E. coli* W3110 for D-lactic acid production. Alternating periods of rational pathway engineering and

serial transfer-based metabolic evolution were used to fine-tune strain performance. In this case, the engineered central metabolic pathway enabled co-selection for growth and lactic acid production because the engineered strain was only able to regenerate NAD^+ through the production of lactic acid. The final strain was able to produce more than 500 mM D-lactic acid with 98% purity from mineral salts medium containing 5% glucose [133].

E. coli KO11 was modified for D-lactate production by alternating rounds of rationally-selected genetic manipulations and directed evolution. This re-engineering included elimination of the ethanol pathway, along with other competing pathways. This pathway elimination left the lactate-producing pathway as the sole route for regenerating NAD^+. Then, the engineered strain was subjected to sequential transfer-based evolution. By co-selecting growth and lactate production, the resulting strain SZ132 was able to produce 1000±20 mmol D-lactate in rich medium with 10% (w/v) glucose and 700±11 mmol D-lactate from defined NBS medium, also with 10% (w/v) glucose [134]. SZ132 was further modified to eliminate loss of carbon due to co-product formation. The poor performance of the resulting strain SZ186 in mineral salts medium motivated further rounds of serial transfer-based metabolic evolution to improve the ability to completely utilize glucose [135]. 1.23±0.03 M D-lactate could be produced by the final strain SZ194 from NBS medium with 12% (w/v) glucose.

A similar strategy was used for succinate production. *E. coli* ATCC 8739 was rationally engineered for succinate production by the traditional elimination of competing pathways. In this case, cell growth was coupled with succinate production since the succinate-synthesizing pathway was the sole route for regeneration of NAD^+. With the alternating use of metabolic engineering and evolution for more than 1,000 generations, the resulting strain HX024 was able

to produce 813±28 mM succinate from NBS mineral salts medium with 12% (w/v) glucose, which was 4.5 fold more than the parent strain [136].

Similarly, deleting the alternative NAD^+-generating pathways resulted in succinate production as the only route for oxidizing NADH. After conducting 2,000 generations of metabolic evolution, the selected mutants produced 622–733 mM succinate from AM1 medium with 100 g L^{-1} glucose and 516 mM malate from NBS medium with 100 g L^{-1} glucose [137].

The examples described above were all able to redesign the central metabolic pathways so that the biocatalyst could only maintain redox balance by producing the target compound. In this manner, cells that acquired mutations that enabled increased production of the target compound would also have increased ability to turn over NADH, resulting in increased biomass production and growth. There are also examples of metabolic pathway redesigns so that production of the target compound is the only way to produce the ATP needed for growth.

For example, the L-lactic acid producer SZ194 described above was re-engineered for alanine production by replacing the *ldhA* gene with the gene encoding an alanine dehydrogenase from *Geobacillus stearothermophilus* [138]. In the redesigned metabolic pathways, cells could produce ATP by producing alanine. Through a similar alternating strategy of rational engineering and serial transfer-based evolution, the resulting strain could convert 120 g L^{-1} of glucose to 1279 mmol L-alanine within 48 h.

A similar strategy was used to engineer *E. coli* for production of optically pure L-lactic acid. *E. coli* SZ63 [133], initially engineered for D-lactate production, was modified for L-lactate production by replacing the native *ldhA* gene with the *ldhL* gene from *Pediococcus acidilactici* [139]. Metabolic evolution was then required to improve the expression of the foreign gene, as determined by comparison of growth and lactate dehydrogenase activity to the L-lactic acid

producing parent strain. After a period of evolutionary selection, the selected strain SZ85 was able to produce 505±12 mM L-lactate from M9 medium with 5% (w/v) glucose within 24h.

Computational strain design via the OptKnock optimization algorithm was used to predict manipulations needed for lactic acid production by *E. coli* strain MG1655 [140]. Growth of this engineered strain was coupled to lactate production and thus approximately 1,000 generations of adaptive evolution in minimal media followed the strain engineering. Eleven different strains were characterized by this method. The best lactate producer attained a titer of 19 mM lactate from 0.2% glucose.

Directed evolution was also applied to biocatalysts in conjunction with targeted genetic manipulations to enhance strain performance. An industrial *S. cerevisiae* strain was modified for production of ethanol from pentose sugars by introducing the D-xylose-utilization cassette from *Clostridium phytofermentans*. This engineered strain was then subjected to both treatment with the chemical mutagen ethyl methanesulfonate (EMS) and genome shuffling. The EMS treatment aimed to increase the mutation rate and the genome shuffling provided large-scale rearrangements that were unlikely to occur naturally. The resulting strain was then subjected to directed evolution in the form of 11 sequential batch cultures in the presence of increasing concentrations of xylose. The initial xylose concentration was 40 g L^{-1}, the final concentration was 100 g L^{-1}. The final strain produced ethanol at a yield of 0.46 g g^{-1} in YP medium from a mixture of xylose and glucose. This yield was double that observed for the parent strain [141].

Another example of evolutionary improvement of *yeast* behavior is the improvement of lactose utilization by *S. cerevisiae* T1 [142]. After serial transfers in batch fermenters, the ethanol production from lactose was increased from 7.1±0.8 g L^{-1} to 10.52±0.04 g L^{-1}.

Enabling Increased Production by Increasing Tolerance

As described above, it is sometimes possible to co-select for increased production of a target compound and growth. This enables evolution-based selection of strains with the ability to produce the target compound at increased yield, titer and/or productivity. But such selection schemes are not always feasible. In such cases, strains are often evolved for increased tolerance of an exogenously supplied target compound, with the goal of increasing the strain's ability to produce the target compound. This has met with mixed results (Table 4).

Various projects have aimed to increase the ethanol tolerance of ethanologenic *E. coli* strains through directed evolution. Recombinant KO11 grew poorly in the presence of 35 g L^{-1} ethanol and had a maximum observed ethanol titer of more than 50 g L^{-1}. Its evolved derivative LY01 could tolerate up to 50 g L^{-1} of ethanol and was able to produce 60 g L^{-1} ethanol from 140 g L^{-1} of either glucose or xylose within 72 hrs [75]. Note that the evolution of strain LY01 included selection for high ethanol-producing strains, as evaluated by colony size and morphology on solid media. Strain SZ470, the evolved derivative of nontransgenic *E. coli* KC01 [73], not only had increased ethanol tolerance but also had improved ethanol production. Specifically, within 72 hours SZ470 produced 23.5 g L^{-1} ethanol from 50 g L^{-1} xylose; KC01 only produced 16 g L^{-1}.

A non-carboxylic acid producing derivative of *E. coli* strain MG1655 was evolved for tolerance to exogenously supplied octanoic acid. When the evolved strain LAR was provided with the thioesterase for octanoic acid production, the observed octanoic acid titer was approximately twice as high as that observed for the parent strain, also encoding the thioesterase [112].

In one of the first demonstrations of gTME, TATA-binding protein Spt15 of *S. cerevisiae* was targeted, with the goal of improving tolerance to 20% (v/v) ethanol and up to 120 g L^{-1} glucose [79]. The selected mutant strain had an ethanol yield of 0.40 g/g, an increase of 15% relative to the parent.

However, increased tolerance does not always contribute to improved production. This disconnect between tolerance of exogenously supplied inhibitor and increased ability to produce the inhibitor has also been observed in some rational engineering-based design schemes [38]. *E. coli* strain SA481 was obtained by evolution of an isobutanol-producing strain for tolerance to exogenously supplied isobutanol [102]. However, the isobutanol titer of 20 g L^{-1} achieved by this evolved strain in standard fermentation conditions was similar to the parent.

Reverse Engineering of Evolved Strains

As is evident from the cases described above, evolution-based strain improvement is a valuable partner for rational engineering strategies. Our reliance on evolutionary techniques is partially due to incomplete understanding of our biocatalysts. Investing the time and effort to fully characterize the evolved strains and identify and understand the mutations that confer evolved strain behavior can increase our understanding of these organisms (Table 5). This increased understanding can in turn lead to decreased dependence on evolutionary methods. We refer to this characterization of evolved strains as reverse engineering.

Recently, characterization tools such as genome sequencing, transcriptome analysis and metabolic flux analysis, have become faster and cheaper, all of which support reverse engineering efforts. Reverse engineering aims not to identify all mutations that occurred during evolution, but to instead identify the crucial mutations which enable the evolved phenotype, and to understand how these mutations are beneficial. Reverse engineering is a tool to expose the

story behind the evolution experiments, which can in turn accelerate the pace of future biorenewable fuels and chemicals design processes.

Identifying and understanding the key mutations that support the evolved phenotype requires knowledge of what mutations occurred during the evolutionary engineering. Whole-genome sequencing is invaluable to this goal [143]. As described above, isobutanol-producing *E. coli* was subjected to directed evolution to increase both isobutanol tolerance and isobutanol production [102]. The evolved strain, SA481, was found to have acquired one single nucleotide change, 25 insertion sequences and a genomic deletion involving 62 genes. 28 of these mutations were individually reconstructed in the parent strain and tested for their contribution to the evolved phenotype. Five of these mutations were found to contribute to the evolved phenotype. These five mutations, essentially deletion of *acrA*, *gatY*, *tnaA*, *yhbJ* and *marCRAB*, were then combined into a single strain TW306 and the resulting phenotype was assessed. The engineered strain TW306 reproduced the evolved strain phenotype [102].

After finding the important mutations, the next step is to explore which mutations promote fitness, the mechanisms of how the tolerance to inhibitors has increased, and the functions of poorly-characterized enzymes and pathways involved in the evolved phenotype. In addition to the use of genome sequence as described above, transcriptome analysis and metabolic flux analysis have proven quite useful in such efforts [43,122].

For the ethanologenic *E. coli* strain evolved for furfural tolerance, transcriptome analysis led to the discovery that YqhD is the primary NADPH-dependent furfural reductase in *E. coli* [122,144]. Sequence analysis of the region surrounding *yqhD* identified an insertion mutation within *yqhC* in the evolved strain. This mutation was found to be responsible for the decreased expression of YqhD because YqhC is a regulator of *yqhD* [145].

E. coli strain MG1655 was subjected to evolution for increased ethanol tolerance and then characterized via a variety of techniques, including genome sequencing, transcriptome analysis and ribosome profiling. Six mutated genes (*metJ, rho, rpsQ, ispB, lptF, topA)* were identified in evolved strain MTA156. Only one of these mutations, H122L within *topA*, was found to have no impact on ethanol tolerance. The main goal of this study was to understand the impact of ethanol on gene expression machinery, and thus these researchers focused the rest of their characterization on the MetJ transcriptional repressor, Rho transcription termination factor, and RpsQ ribosomal subunit protein. The H31P mutation in RpsQ was found to decrease the effect of ethanol on miscoding errors during translation. This decrease in translational misreading was due to enhanced ribosome accuracy. The *rho* [L270M] mutation led to decreased activity of Rho-dependent terminators, where these terminators can cause abnormal transcription termination under ethanol stress. The ΔE91mutation within MetJ results in increased expression of methionine biosynthesis genes; this presumably leads to increased methionine availability, preventing some ribosomal stalling [68]. The thorough reverse engineering efforts employed here have provided multiple new mechanisms of ethanol toxicity, as well as design strategies for mitigating this toxicity.

As described above, a metabolic pathway was designed in *E. coli* C so that production of succinate was linked to growth. It was found that in this evolved strain, the glucose uptake system had switched from the traditional PEP-based phosphotransferase system to the GalP permease. This change occurred via a mutation in *ptsI* and allowed more PEP to be directed towards succinate, instead of being converted to pyruvate via glucose import. A second change in the evolved strain was the increased usage of the PEP carboxykinase (Pck) pathway to convert PEP to OAA instead of the PEP carboxylase (Ppc) pathway. The Pck pathway produces a

molecule of ATP for every conversion of PEP to OAA while the PPC pathway does not. This results in the production of more ATP in the evolved strain. This change in pathway usage was evidenced by both increased activity of the Ppc pathway and increased expression of the genes encoding this pathway. This increased expression was attributed to at least three distinct mutations. These results demonstrate the ability to identify important mutations and pathway changes even in the absence of whole-genome sequence data [146].

A distinct strain of *E. coli* engineered for succinate production, NZ-037, was also subjected to metabolic evolution to improve its succinate production. The evolved strain, HX024, was found to have three distinct single base pair changes within the *lpdA* gene. Each of these nucleotide changes resulted in a change in the corresponding amino acid. This alteration to the LpdA protein sequence eliminated the NADH sensitivity of the pyruvate dehydrogenase enzyme complex, of which LpdA is a component. This decreased NADH sensitivity enabled increased succinate production [136].

Eleven *E. coli* strains subjected to computationally-designed engineering strategies and then evolved by co-selecting for growth and lactate production were subjected to whole-genome sequencing and characterization [147]. Seven of these sequenced strains were found to contain an 82-bp deletion mutation in the *rph-pyrE* region. *rph* encodes an RNase and *pyrE* encodes an enzyme involved in biosynthesis of pyrimidine nucleotides. Introduction of this mutation into the parent strain was shown to increase growth by approximately 15%.

These examples demonstrate how reverse engineering efforts can reveal novel design strategies and increase our understanding of microbial physiology and metabolism.

Modeling of Evolution

There has been much work performed recently in modeling the evolutionary process *in silico*. This work is discussed here only briefly. A thermodynamic perspective has also been applied to these models and, as supported by experimental data of *Thermoanaerobacterium saccharolyticum* evolution, shown that the rate of entropy production increases as a strain is allowed to evolve [148]. This entropy increase was shown to be asymptotically approaching a maximum rate of entropy production predicted according to the Boltzmann distribution. Further consideration of this evolved organism in terms of metabolic control analysis successfully predicted the enzyme likely to be altered during the evolution of *T. saccharolyticum* in serial cultures [149]. Specifically, the metabolic reaction performed by phosphoglucose isomerase had the large flux control coefficient of the various metabolic reactions and when sequenced in 10 evolved clones, the gene encoding phosphoglucose isomerase was found to contain a variety of mutations. These results demonstrate the ability of a predictive model to prioritize targets for engineering, targeted enzyme evolution, or analysis when characterizing evolved isolates.

Concluding Statement

We have summarized the use of serial dilution and engineering evolutionary methods to improve the production of biorenewable fuels and chemicals. These evolutionary tools have proven quite effective and as reverse engineering tools become more accessible, additional design strategies can be distilled from these evolved strains.

Tables

Table 1. Representative mutations within the *E. coli* genome.

Location	Type	Possible Impacts	Example	Ref.
coding	"Silent" single nucleotide change where the amino acid sequence is not changed	Transcription and translation rate, protein folding	Silent mutations in *ompA* impacted transcription and translation. Silent mutations in recombinant EgFABP1 gene affected translation and protein folding.	[150, 151]
coding	Non-synonymous; single nucleotide change where amino acid sequence is altered	Transcription and translation rate, enzyme activity	Single point mutation in *rho* decreased the activity of Rho-dependent terminators. Three single point mutations in *lpdA* eliminated NADH sensitivity.	[68, 136]
noncoding	Single nucleotide changes	Transcription and translation of nearby genes	Four different single point mutations in the *mgl* operator (*mglO)* increased the glucose transport rate.	[152]
coding	Insertion	Silencing or increased expression of the affected gene and possible downstream genes	An insertion mutation in *hfq* led to significant pleiotropic phenotypes, such as increased cell size and decreased growth rates. An IS10 insertion mutation in *yqhC* affected the transcription of downstream genes *yqhD* and *dkgA*.	[145, 153]
noncoding	Insertion or Deletion	Perturbed expression of nearby genes	Insertion mutations upstream of the *bgl* promoter activated the silent *bgl* operon. Deletion mutation in the *mgl* operator (*mglO)* increased the glucose transport rate.	[152, 154]
coding	Deletion	In-frame deletions can alter enzyme activity. Out-of-frame deletions can result in translation termination and altered or absent enzyme activity.	The single-amino acid deletion in *metJ* altered MetJ activity, resulting in increased expression of the methionine biosynthesis pathway.	[68]

Table 2. The use of directed evolution to increase tolerance to inhibitory products or inhibitors in biomass-derived sugars.

Strain	Inhibitory compound	Tolerance	Duration of evolution	% increase compared to parent strain	Ref.
E. coli SA481	Isobutanol	0.8% (w/v)	45 transfers	100	[102]
E. coli G3.2	Isobutanol	2% (w/v) (glucose)	500 generations	60	[103]
E. coli X3.5	Isobutanol	1.75% (w/v) (xylose)	430 generations	40	[103]
S. cerevisiae JBA-mut	Isobutanol	1.9% (v/v)	30 transfers		[105]
	2-butanol	3% (v/v)	30 transfers	58	[105]
	1-butanol	1.9% (v/v)	30 transfers		[105]
P. putida JUCT1	Cyclohexane	60% (v/v)	12 transfers	1100	[106]
	Decalin	60% (v/v)	12 transfers		[106]
	Methyl cyclohexane	60% (v/v)	12 transfers		[106]
	Toluene	60% (v/v)	12 transfers		[106]
E. coli EMFR9	Furfural	0.13% (w/v)	54 transfers	160	[122]
	5-HMF	0.25% (w/v)	54 transfers		[126]
S. cerevisiae RK60-5	Simulated spruce hydrolysate	60% (w/v)	429 generations	200	[127]
S. cerevisiae KE1-17	Spruce hydrolysate	50% (v/v)	97 generations	150	[127]

Table 3. The use of directed evolution to increase production of biorenewable fuels and chemicals.

Strain	Product	Medium	Production by Evolved Strain	Ref.
E. coli KO12	Ethanol	Luria Broth + 100 g L^{-1} glucose	1.2 M	[131]
E. coli KO11	Ethanol	Luria Broth + 80 g L^{-1} xylose	0.90 M	[131]
E. coli TCS009 e50rep1/pLOI297	Ethanol	Defined minimal medium + 40 g L^{-1} glycerol	0.39 M	[132]
S. cerevisiae GS1.11-26	Ethanol	YP + 35 g L^{-1} xylose	0.46 g g^{-1}	[141]
S. cerevisiae T1-E	Ethanol	Defined minimal medium + 25 g L^{-1} lactose	0.22 M	[142]
E. coli SZ63	D-lactic acid	M9 + 50 g L^{-1} glucose	0.54 M	[133]
E. coli SZ132	D-lactic acid	Luria Broth + 100 g L^{-1} glucose	1.0 M	[134]
E. coli SZ132	D-lactic acid	NBS + 100 g L^{-1} glucose	0.70 M	[134]
E. coli SZ194	D-lactic acid	NBS + 120 g L^{-1} glucose	1.2 M	[135]
E. coli SZ85	L-lactic acid	M9 + 50 g L^{-1} glucose	0.51 M	[139]
E. coli HX024	Succinate	NBS + 120 g L^{-1} glucose	0.81 M	[136]
E. coli KJ060	Succinate	AM1 + 100 g L^{-1} glucose	~0.70 M	[137]
E. coli KJ071	Malate	AM1 + 100 g L^{-1} glucose	0.52 M	[137]
E. coli XZ132	L-alanine	AM1 + 120 g L^{-1} glucose	1.3 M	[138]

Table 4. Increased tolerance leading to increased production.

Strain	Medium	Production	% increase relative to parent strain	Ref.
E. coli LY01	Luria Broth + 140 g L^{-1} glucose	61±3 g L^{-1} ethanol	42.8	[75]
E. coli LY01	Luria Broth + 140 g L^{-1} xylose	63±1 g L^{-1} ethanol	42.8	[75]
E. coli SZ470	Luria Broth + 50 g L^{-1} xylose	23.5 g L^{-1} ethanol	100	[73]
E. coli LAR1 + pJMY-EEI82564	Luria Broth + 15 g L^{-1} glucose	540±50 mg L^{-1} carboxylic acid	400	[112]
S. cerevisiae spt15-300	Minimal medium + 100 g L^{-1} glucose	0.40 g ethanol g^{-1} glucose	*n/a*	[79]

Table 5. Examples of reverse engineering projects involving *E. coli*.

Motivation	Characterization Method	Results	Ref.
Increasing isobutanol tolerance and production	Whole-Genome Sequencing	Silencing of *acrA*, *gatY*, *tnaA*, *yhbJ* and *marCRAB* enables increased isobutanol tolerance.	[102]
Increasing furfural tolerance	Transcriptome Analysis, Targeted Sequencing	YqhD is the primary NADPH-dependent furfural reductase; YqhC is a regulator of *yqhD*	[122, 145]
Understand the impact of ethanol on gene expression machinery	Genome Sequencing, Transcriptome Analysis, Ribosome Profiling	RpsQ [H31P]: decreased the effect of ethanol on miscoding errors during translation; Rho [L270M]: decreased activity of Rho-dependent terminators; MetJ [ΔE91]: increased expression of methionine biosynthesis gene	[68]
Increasing succinate production	Genome Sequencing, Targeted Enzyme Assay, Real-Time Reverse Transcription-PCR	A point mutation in *ptsI* allows more PEP to be directed towards succinate; Increased usage of Pck pathway instead of Ppc pathway enables increased ATP production.	[146]
Increasing succinate production	Genome Sequencing; Transcriptome Sequencing; Targeted Enzyme Assay;	Mutations in *lpdA* eliminated the NADH sensitivity of the pyruvate dehydrogenase enzyme complex, which enabled increased succinate production.	[136]

Figures

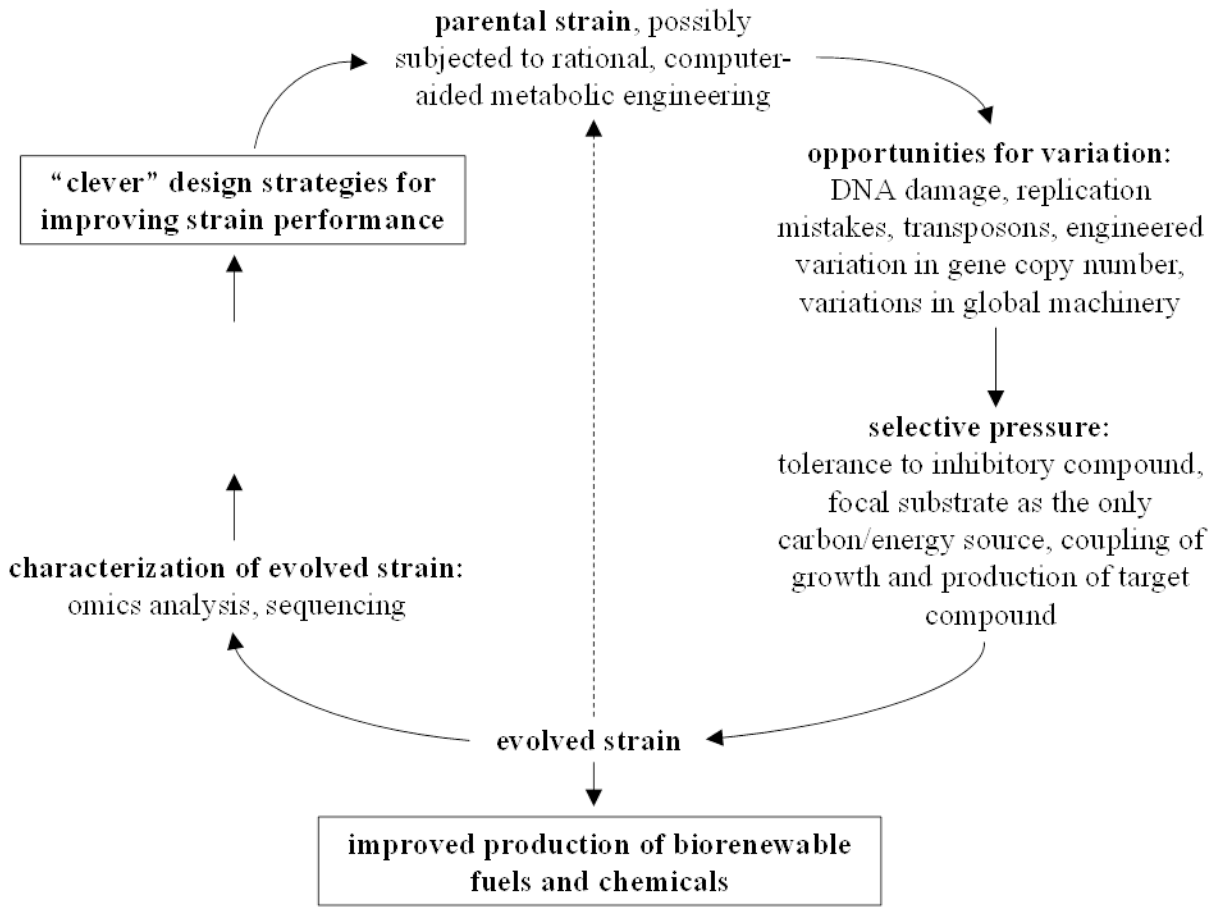

Figure 1. Evolutionary strain improvement can play an important role in the strain design cycle. Characterization of the evolved strain and its sequence and expression changes can yield novel design strategies for future improvement.

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CHAPTER 3. EVOLUTION FOR EXOGENOUS OCTANOIC ACID TOLERANCE IMPROVES CARBOXYLIC ACID PRODUCTION AND MEMBRANE INTEGRITY

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Abstract

Carboxylic acids are an attractive biorenewable chemical, but as with many biorenewables, their toxicity to microbial biocatalysts limits their fermentative production. While it is generally accepted that membrane damage is the main mechanism of fatty acid toxicity, previous metabolic engineering efforts that increased membrane integrity did not enable increased carboxylic acid production. Here we used an evolutionary approach to improve tolerance to exogenous octanoic acid, with the goal of learning design strategies from this evolved strain. This evolution of an *Escherichia coli* MG1655 derivative at neutral pH in minimal media produced a strain with increased tolerance not only to octanoic acid, but also to hexanoic acid, decanoic acid, n-butanol and isobutanol. This evolved strain also produced carboxylic acids at a 5-fold higher titer than its parent strain when expressing the Anaerococcus tetradius thioesterase. While it has been previously suggested that intracellular acidification may contribute to carboxylic acid toxicity, we saw no evidence that the evolved strain has increased resistance to this acidification. Characterization of the evolved strain membrane showed that it had significantly altered membrane polarization (fluidity), integrity (leakage) and composition relative to its parent. The changes in membrane composition included a significant increase in average lipid length in a variety of growth conditions, including 30 °C, 42 °C, carboxylic acid

challenge and ethanol challenge. The evolved strain has a more dynamic membrane composition, showing both a larger number of significant changes and larger fold changes in the relative abundance of membrane lipids. These results highlight the importance of the cell membrane in increasing microbial tolerance and production of biorenewable fuels and chemicals.

Introduction

Carboxylic acids are an attractive biorenewable chemical, due in part to their ability to be catalytically converted to a variety of industrial chemicals, such as alkanes (Lennen et al., 2010) and olefins (Lopez-Ruiz and Davis, 2014). Significant strides have been made in engineering *Escherichia coli* and *Saccharomyces cerevisiae* for production of these compounds (Lennen and Pfleger, 2012, Choi and Da Silva, 2014, Leber and Da Silva, 2014 and Tee et al., 2014). Unfortunately, the toxicity of these carboxylic acids to the microbial biocatalyst appears to be limiting biocatalyst performance. There is strong evidence that membrane damage is the main mechanism of toxicity (Lennen et al., 2011, Jarboe et al., 2013 and Liu et al., 2013), including the observation that membrane leakage sharply increases near the end of the production phase (Royce et al., 2013). Previous metabolic engineering efforts that were successful in increasing the membrane integrity were not successful in increasing carboxylic acid titers (Lennen and Pfleger, 2013), though other efforts that increased tolerance by preventing medium-chain fatty acid incorporation into the cell membrane did enable increased production (Sherkhanov et al., 2014).

Product toxicity is frequently encountered in metabolic engineering projects (Nicolaou et al., 2010 and Jarboe et al., 2011). There are a variety of strategies for mitigating this toxicity, including in situ removal of the product during the course of the fermentation (Dafoe and Daugulis, 2014, Lopez-Garzon and Straathof, 2014 and McKenna et al., 2014) and increasing the

robustness of the microbial biocatalyst. Biocatalyst tolerance can be increased either through an evolutionary approach (Portnoy et al., 2011 and Abatemarco et al., 2013) or through rational engineering that addresses known mechanisms of toxicity (Luo et al., 2009 and Jarboe et al., 2011). The evolutionary approach has been quite successful in improving strain performance when production of the target compound can be linked to growth (Jarboe et al., 2010). This linkage between growth and production generally requires redox balance and a net ATP production by the pathway. Evolution for tolerance to an exogenously-supplied inhibitor with the goal of increasing production of that same inhibitory compound has met with mixed results (Yamane et al., 1998, Atsumi et al., 2010 and Wang et al., 2011).

The use of evolutionary-based strain improvement relies on Orgel′s Second Rule that "evolution is cleverer than you are" (Dennett, 1996). In order to learn from this cleverness and increase our arsenal of rational strategies for strain improvement, it is important to reverse engineer these evolved strains. The outcome of such reverse engineering efforts have included the identification of improved succinate production pathways (Zhang et al., 2009), the identification of the major *E. coli* furfural reductase (Miller et al., 2009), methods of enabling ethanol resistance in *E. coli* (Haft et al., 2014) and methods for improving the *yeast* membrane for thermotolerance (Caspeta et al., 2014).

Here we describe the evolution of *E. coli* for tolerance to exogenously supplied octanoic acid, with a resulting 5-fold increase in carboxylic acid production and increased tolerance not just of carboxylic acids, but also of butanol isomers. This work describes the phenotypic characterization of this strain, with a particular focus on the membrane.

Methods

Strains and growth conditions

E. coli strains used in this study are listed in Table 1. Carboxylic acid stock solutions were prepared in 100% ethanol. The concentration of ethanol used in these stock solutions had no significant impact on growth (data not shown). Media were adjusted to pH 7.0 with 2.0 M potassium hydroxide.

Directed evolution and strain selection

Strain ML115 was subjected to 17 serial transfers in 350 mL MOPS medium (Wanner, 1994) with 2.0% dextrose and 350 mg/L chloramphenicol in 500 mL fleakers. The temperature was controlled at 37 °C and mixing was maintained at 150 rpm with magnetic stir bars. The pH was maintained at 7.0 using gravity-fed 2.0 M potassium hydroxide. The octanoic acid concentration was periodically increased in 10 mM increments. The final culture was plated onto solid media and individual colonies were selected and characterized by measuring the specific growth rate in closed fermentation vessels containing MOPS 2.0 wt% dextrose with 10 mM C8, pH 7.0, 150 rpm, 37° C. Colonies were first analyzed as mixed cultures of 5–6 strains. The strains populating the mixed population showing the highest specific growth rate were then characterized in pure culture. This work describes the characterization of the strain with the highest growth rate, LAR1.

Strain characterization

Tolerance to hexanoic, octanoic and decanoic acid was performed in 500 mL fleakers automatically controlled at 37 °C, 150 rpm and pH 7.0 in 350 mL MOPS 2.0% dextrose. Tolerance to n-butanol and isobutanol was assessed in 250 mL baffled flasks with 25 mL MOPS 2.0 wt% dextrose, 37 °C, 200 rpm, with an initial media pH of 7.0.

Plasmid construction

The acyl–acyl carrier protein thioesterase (acyl-ACP TE) from *Anaerococcus tetradius* (GenBank: EEI82564) which primarily produces octanoic acid (Jing et al., 2011) was provided by Dr. Nikolau at Iowa State University. The acyl-ACP TE originally cloned in pUC57 was digested with BamHI and EcoRI at 5′ and 3′ ends and cloned into pTrcHisB plasmid (Life Technologies) to produce plasmid pJMY-EEI82564.

Characterization of carboxylic acid production

Strains transformed with pJMY-EEI82564 were spread onto Luria Broth (LB) plates with 100 mg/L ampicillin and incubated at 30 °C overnight. Individual colonies were precultured in 5 mL LB media with 100 mg/L of ampicillin in 50 mL centrifuge tubes overnight, and then were inoculated into 250 mL shake flasks containing fresh 50 mL of LB media with 1.5% dextrose, 100 mg/L ampicillin, and 1.0 mM of isopropyl-β-d-thiogalactopyranoside (IPTG). Cultures were grown at 30 °C on a rotary shaker at 200 rpm. The initial ODs were approximately 0.07 and the cells were harvested after 72 h.

Determination of carboxylic acid titers

Carboxylic acid production was quantified by an Agilent 7890 gas chromatograph equipped with an Agilent 5975 mass spectroscope using flame ionization detector and mass spectrometer (GC-FID/MS) after carboxylic acid extraction as described in (Zhang et al. 2012). Briefly, 1 mL of whole liquid media was taken and extracted by 9 mL of a mixture of chloroform/methanol/water (1.5:1.0:1.0 vol) with 100 μl acetic acid and 20 μL of a mixture of internal standards (1.0 mg/mL for each). The organic phase (bottom) was filtered through a glass column filled with 1.0 g of magnesium sulfate, and then dried under nitrogen flow. After methylation with 4.0 mL of 5.0% sulfuric acid in methanol at 90 °C for 2 h, 2.0 mL of

hexane/chloroform (4.0:1.0 vol) and 2.0 mL of 0.90% sodium chloride solution were added and vortexed. The top organic layer was filtered through a glass column filled with 0.20 g sodium bicarbonate and 1.0 g of magnesium sulfate, dried again with nitrogen gas, and then resuspended in 500 μL of hexane before injection to GC-FID/MS. The temperature for GC-FID/MS analysis was held at 50 °C for 1 min, ramped to 140 °C at 20 °C/min, and then to 220 °C at 4 °C/min before it was raised to 280 °C at 15 °C/min. Helium was used as a carrier gas and the flow rate was 1 mL/min through a DB-5MS separation column (30 m, 0.25 mm ID, 0.25 μm, Agilent). Nonanoic (C9), tridecanoic (C13), pentadecanoic (C15), and heptadecanoic (C17) acids were added as internal standards for carboxylic acid quantification.

Membrane characterization

Membrane permeability was assessed by SYTOX Green staining. Strains were grown to OD550 of ~1 in MOPS 2.0 wt% dextrose at 37 °C and 200 rpm, pelleted, washed twice, and then resuspended in PBS pH 7.0 to OD550 1. The washed cells were treated either with 10 mM octanoic acid at pH 7.0 or a similar volume of PBS pH 7.0. Cells were incubated at 37 °C and 200 rpm for 90 min and then diluted 10-fold into PBS pH 7.0. SYTOX Green Nucleic Acid Stain (Invitrogen) was added from a 5.0 mM stock in DMSO to a final concentration of 5.0 μM. After 15 min of staining, cells were subjected to analysis using a BD Biosciences FACSCanto II flow cytometer with simultaneous measurements of forward and side laser scatter at 488 nm excitation and 530 nm emission. 15,000 cells were collected per sample.

Membrane fluidity characterization was performed using 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) accordingly to previously-described methods (Royce et al., 2013). Briefly, cells were grown to mid-log in MOPS 2.0% dextrose at 37° C, harvested, washed and resuspended in PBS pH 7.0 at a final concentration of 1×108 cells/mL with 0.2 μM DPH. Fluorescence polarization

was determined based on vertical and horizontal fluorescence readings obtained using a Synergy 2 Multi-Mode plate reader. The impact of temperature was assessed by changing the temperature at which the assay was performed.

The membrane lipid composition was analyzed using a modified Bligh and Dyer method, as previously described (Royce et al., 2013). This protocol differs from the carboxylic acid production analysis in that it uses cells collected from the pelleting of 25 mL of culture media, while the carboxylic acid titer analysis featured 1 mL of sample containing both media and cells. Cells for the composition analysis were grown in MOPS 2.0 wt% dextrose with an initial pH of 7.0 in baffled flasks and shaking at 200 rpm from an initial OD550 of 0.1 to mid-log (OD550 ~0.8) prior to harvesting.

Intracellular pH measurements

The intracellular pH was measured using a pH-dependent green fluorescent protein encoded by pJTD1 as previously described (Thomas et al., 2001 and Royce et al., 2014). Briefly, cells were grown in LB media at an initial pH of 7.0 with 100 mg/L ampicillin and 200 μM l-arabinose from an initial OD550 of 0.1 to mid-log (OD550 ~0.8), harvested by centrifugation and resuspended in PBS pH 7.0. Samples for the standard curve were treated with sodium benzoate. Experimental samples were treated with octanoic acid at pH 7.0. The fluorescence intensity was measured at 30 °C using excitation filter=485/20 nm and emission filter=528/20 nm.

Results

Though metabolic engineering efforts for carboxylic acid production have been able to effectively increase titers and yields, it appears that product toxicity limits further gains in titer (Lennen et al., 2011, Lennen and Pfleger, 2012, Jarboe et al., 2013 and Tee et al., 2014). Here we

used directed evolution to improve *E. coli* tolerance to octanoic acid. This evolution provides a system for study and characterization, so that we can identify design strategies for further increasing tolerance in production strains.

Development and isolation of evolved strains

An evolved strain exhibiting an octanoic acid tolerance phenotype was obtained by sequentially transferring parent strain ML115 in MOPS minimal media containing octanoic acid while maintaining the media pH at 7.0 (Fig. 1A). Strain ML115 lacks the fatty acid β-oxidation pathway via deletion of *fadD*; this prevents degradation of carboxylic acids. The selective pressure of octanoic acid was increased in 10 mM (1.44 g/L) increments to a final concentration of 30 mM over the course of 714 total hours.

Twenty-six distinct strains were isolated as single colonies from this final mixed population. These 26 strains were grouped into five sets of mixed cultures that each contained five or six distinct strains. These mixed cultures were characterized in the presence of 10 mM C8, with the assumption that mixed cultures containing strains with increased tolerance will show increased growth. These mixed cultures showed specific growth rates ranging from 0.54 to 0.65 h^{-1} (Table 2), relative to the 0.28 h^{-1} shown by the parent. The five strains that composed the mixed culture with the highest specific growth rate were then tested individually and found to have specific growth rates ranging from 0.60 to 0.69 h^{-1}. This work focuses on phenotypic characterization of the evolved strain with the highest growth rate, which we have named LAR1. Genomic sequence analysis of this strain is in progress and will be discussed in a forthcoming publication.

Evolution for octanoic acid tolerance extends to other carboxylic acids and butanol

As expected from the directed evolution process, the evolved strain shows increased C8 tolerance relative to its parent (Fig. 1B). Specifically, when characterized in pure cultures in closed fermentation vessels maintained at pH 7.0, the evolved strain shows only slight growth inhibition during exponential phase from 10 mM C8, though the final OD550 reached during stationary phase (1.4±0.1) is lower than its growth under the control condition (3.2±0.4). Under this same condition, the parent strain grown in the presence of 10 mM C8 shows a growth lag of approximately 10 h and after 24 h has only reached a final OD550 of 0.20±0.03. The evolved strain also shows increased tolerance to 20 mM C8 in shake flasks. Specifically, the parent strain had a specific growth rate of 0.137±0.003 h^{-1} and the evolved strain had 2.2-fold higher rate of 0.30±0.03 h^{-1}.

We have previously observed between 25% and 30% inhibition of MG1655 growth by 10 mM hexanoic acid (C6), C8 and decanoic acid (C10) under this growth condition (Royce et al., 2013), where our parent strain ML115 is an engineered derivative of MG1655 (Table 1). However, our evolved strain showed only very slight inhibition in the presence of 10 mM of each of these three compounds (Fig. 1C). The evolved strain's specific growth rate in the control condition was 0.60 h^{-1}, while the specific growth rate was 0.57, 0.58 and 0.57 h^{-1} in the presence of 10 mM C6, C8 and C10, respectively. This indicates that the useful mutations acquired during evolution for C8 tolerance also apply to C6 and C10. This is consistent with the expectation that saturated, straight-chain carboxylic acids of various lengths have generally similar mechanisms of inhibition.

The evolved strain shows increased tolerance not just of carboxylic acids, but also to two of the most popular butanol isomers. Specifically, the evolved strain showed significantly

increased growth relative to the parent strain in the presence of 0.60% (v/v) (65 mM) isobutanol and n-butanol. The parent strain had a 37% reduction in specific growth rate in the presence of isobutanol while the evolved strain had a growth rate reduction of only 27%. The increased growth rate shown by the evolved strain was significantly (P=0.0018) higher than the parent strain. The parent strain had a 53% decrease in the specific growth rate in presence of n-butanol, while the evolved strain had a reduction of 46% (P=0.054).

The fact that this evolved strain shows increased resistance to membrane-damaging compounds other than carboxylic acids expands the impact of this work and highlights the importance of understanding the physical changes that enable this increased tolerance.

Evolution for octanoic acid tolerance enabled increased octanoic acid production

When metabolic pathway design enables co-selection for increased growth and increased production of the target compound, evolutionary schemes have proven quite effective in improving strain performance, as seen for *E. coli* in regards to production of, for example, ethanol, lactic acid and succinate (Zhang et al., 2009 and Jarboe et al., 2010). However, engineering or evolving for increased tolerance of exogenously-supplied compounds with the aim of improving production has been met with mixed results. Such an approach was effective for ethanol (Yamane et al., 1998 and Wang et al., 2011), but not for isobutanol (Atsumi et al., 2010). Additionally, rational membrane engineering efforts that were able to mitigate carboxylic acid toxicity did not result in increased carboxylic acid production (Lennen and Pfleger, 2013).

However, in the work presented here, the strain evolved for increased octanoic acid tolerance produced significantly higher titers of octanoic acid and nearly all other carboxylic acids when provided with the A. tetradius thioesterase (Fig. 2A). Specifically, the evolved strain produced 180±30 mg/L (1.3±0.2 mM) octanoic acid and 540±50 mg/L (3.0±0.4 mM) total

carboxylic acids, relative to the 32±7 (0.22±0.05 mM) and 100±20 (0.5±0.1 mM) produced by the parent strain. The only carboxylic acid that was not produced at significantly higher amounts by the evolved strain is C18:0, with titers of 8±2 in the evolved strain and 5±2 mg/L in the parent strain. The OD500 value for the evolved strain was an average of 3-fold higher than the parent strain over the 76-hour fermentation (Fig. 2C). Thus, the 5-fold increase in carboxylic acid production cannot solely be attributed to increased biocatalyst abundance.

In addition to producing a significantly higher amount of most carboxylic acids, the evolved strain also shows a significantly altered fatty acid distribution (Fig. 2B). The evolved strain has significantly less C16:0, C18:1 and C18:0 and significantly more C10–OH, C12:1, C12:0 and C14:0 than the parent strain. Surprisingly, the relative abundance of C8:0 is similar for both strains, even though the C8 titers differ by more than two-fold.

The evolved strain has altered membrane properties

It is generally accepted that membrane damage is the key mechanism of microbial inhibition during both carboxylic acid challenge and carboxylic acid production (Lennen et al., 2011, Jarboe et al., 2013, Royce et al., 2013 and Royce et al., 2014). Therefore, we compared the membrane characteristics of the evolved strain to the parent strain.

Carboxylic acid-induced membrane leakage has previously been quantified using both Mg2+ leakage (Liu et al., 2013 and Royce et al., 2013) and permeability to SYTOX (Lennen and Pfleger, 2013). Here we used the SYTOX method, where permeability to this compound indicates a damaged membrane. Flow cytometry was used to separate and quantify SYTOX-permeable and SYTOX-impermeable cells. This analysis showed that the evolved strain showed increased resistance to the membrane-damaging effect of 10 mM C8 during exogenous challenge (Fig. 3A). Specifically, 83% of the parent strain population became SYTOX-permeable while

only 49% of the evolved strain population became permeable. This increased resistance is consistent with the increased tolerance and increased carboxylic acid production.

We previously demonstrated that while transient challenge with exogenous C8 can result in altered membrane fluidity, as indicated by the membrane polarization value, cells given a few hours to adapt to this challenge were able to compensate for this perturbation (Royce et al., 2013). Consistent with this adaptability was the fact that there was no significant change in membrane fluidity during carboxylic acid production (Royce et al., 2013). However, it was observed here that the evolved strain has a significantly higher membrane polarization than the parent strain in the temperature range of 30–45 °C (Fig. 3A). This increased polarization corresponds to an increase in membrane rigidity and decreased fluidity.

The evolved strain has altered membrane composition

This altered membrane integrity and rigidity could be due to altered lipid composition of the membrane. Therefore we grew both strains in a variety of conditions known to impact the membrane composition and measured the various membrane lipid components (Table 3). Note that our concentration of 430 mM ethanol (2.0%) was motivated by published studies of ethanol toxicity (Luo et al., 2009).

Previous membrane engineering efforts have aimed to decrease the saturated:unsaturated (S:U) ratio (Luo et al., 2009 and Lennen and Pfleger, 2013). Consistent with increased tolerance being associated with a decreased S:U ratio, we observed that the evolved strain had a significantly lower S:U ratio than the parent strain when grown in the presence of 30 mM C8 (Fig. 3C). We also observed that the evolved strain had a significantly higher S:U ratio than the parent strain when grown at 42 °C (Fig. 3C, Table 3). Our observed decrease of the S:U ratio in the parent strain in the presence of ethanol is consistent with previous reports (Luo et al., 2009),

as is our observation of a positive relationship between the S:U ratio and growth temperature for the parent strain (Okuyama et al., 1977).

Other membrane engineering efforts have prevented incorporation of medium-chain fatty acids into the membrane via deletion of *aas* (Sherkhanov et al., 2014). In this case, the engineered strain showed decreased C12/C16 and C14/C16 ratios, increased viability during fatty acid production and a 20% increase in fatty acid production when the fermentation was performed with a dodecane overlay for in situ product extraction. Our parent strain and evolved strains had C12/C16 ratios (mol) of 0.05±0.03 and 0.0040±0.0004 and C14/C16 ratios of 0.10±0.03 and 0.051±0.002, respectively. This decrease in both of these ratios during the evolutionary process is consistent with the previously-described engineering efforts, though the ratios obtained by our evolved strain are lower than those obtained via *aas* deletion (Sherkhanov et al., 2014).

In addition to the altered S:U, C12/C14 and C14/C16 ratios, we observed that our evolved strain has a significantly increased average membrane length relative to its parent (Fig. 3D). This increase is consistent with the dose-dependent increase in average length that we previously observed during short-term adaptation to C8 (Royce et al., 2013). In these previous short-term adaptation experiments, *E. coli* MG1655 was able to attain average lipid lengths of up to 16.26. However, the evolved strain is able to attain average lengths of 16.39, while the largest value observed for the parent strain was 16.18. This significant increase in average lipid length for the evolved strain was observed under a variety of conditions, including exogenous ethanol challenge and growth at 42 °C (Fig. 3D).

This increase in average lipid length is an intriguing strategy for potentially increasing membrane integrity. If one desires to mimic this change as a rational metabolic engineering

strategy, a more detailed comparison of the lipid composition of the two strains is useful (Table 3). This comparison shows that for at least four of the five conditions tested, the C16:1 content is significantly lower in the evolved strain and the C16:0, C18:1, C19cyc and total C18 pool are all significantly higher in the evolved strain. Note that the total C18 pool includes C18:1, C18:0 and C19cyc. Thus, the evolved strain seems to have (a) changed the distribution of lipids within the C16 pool and (b) increased the relative size of the C18 pool.

The evolved strain has increased variability in membrane content

The data discussed above involved a direct comparison of the relative membrane composition between the evolved and parent strain. We also considered changes in the membrane composition in response to changes in the growth environment (Table 3). Our observed increase in relative abundance of C18:1 in the parent strain in the presence of ethanol and at 30 °C relative to 37 °C is consistent with previous reports (Ingram, 1982), as is our observed decrease in C18:1 and C19cyc content at 42 °C (Yuk and Marshall, 2003).

The most striking difference between our two focal strains is the observation that the evolved strain has an increased dynamic range of membrane composition. First, the evolved strain has a larger number of significant perturbations to membrane content than the parent strain: 27 relative to 16. Second, the number of lipids whose relative amount significantly changed by at least two-fold is larger in the evolved strain than in the parent. The only significant and greater than two-fold change in the parent strain was a 2.0±0.3-fold increase in C18:0 in the presence of C8. Contrastingly, the evolved strain had four perturbations of at least two-fold: 2.15±0.04 for C16:1 in the presence of C8, −2.2±0.2 for C17cyc in the presence of C8, 8±2 for C12:0 at 30° C and 3.0±0.3 for C18:0 at 30° C. Finally, it should be noted that for two of the four experimental conditions, 2.0% ethanol and 42 °C, at least seven of the eight lipids had a

significantly altered relative abundance in the evolved strain. The highest number of significantly perturbed lipids in the parent strain in any one condition was five, observed in response to octanoic acid challenge.

Thus, the evolved strain appears to be able to implement more, and larger, perturbations to the relative distribution of membrane lipids than the parent strain.

Similar sensitivity to intracellular acidification

Our group has previously demonstrated that exogenous challenge with octanoic acid results in intracellular acidification, even when the bulk media pH is maintained at 7.0 (Royce et al., 2014). Intracellular pH was subsequently monitored during carboxylic acid production and no acidification was observed, though it was not clear if this was due to metabolic differences between exogenous challenge and production or insufficient carboxylic acid titers to achieve the dose necessary for acidification (Royce et al., 2014).

Here we measured the intracellular pH of our parent and evolved strains during exogenous challenge with C8 and no significant difference was observed (Fig. 4). This suggests that it is possible for cells to improve C8 tolerance and production without mitigating the intracellular acidification problem.

Discussion and Conclusions

Here we have described the evolution of *E. coli* for tolerance to the straight-chain, saturated carboxylic acid octanoic acid at neutral pH. This evolution was motivated by our desires to not only enable increased production of these compounds but also to understand the physical changes in the biocatalyst that enable the increased tolerance and production. By understanding the physical changes that occurred, we can understand the “clever” tricks described by Orgel′s Rule and become cleverer in our metabolic engineering strategies.

Our identification and analysis of the mutations acquired by this evolved strain will be discussed in forthcoming publications. It has been confirmed that a single amino acid change at position 419 within the RNA polymerase β′ subunit (*rpoC*) contributes to some, but not all, of the gain in fitness of the evolved strain. This mutation is consistent with the global transcription machinery engineering method (Alper et al., 2006) and with outcomes for other evolutionary studies (Harden et al., 2015). We have also confirmed a single amino acid change as position 82 within BasR, the transcriptional regulator component of BasS/BasR system, which has been implicated in the metal response (Ogasawara et al., 2012). Though the contribution of this mutation to the evolved strain phenotype has not yet been determined, this mutation is consistent with the results of other evolutionary studies related to n-butanol tolerance (Reyes et al., 2012). Other mutations, including the possible duplication of a 10-kb genomic region, are currently being verified and investigated.

Our strain evolved at neutral pH for tolerance to exogenously supplied octanoic acid also showed increased tolerance to other compounds (Fig. 1). The increased tolerance to hexanoic and decanoic acids was not surprising, given the chemical similarity of these compounds to octanoic acid. The increased tolerance to n-butanol and isobutanol was surprising, as these compounds are alcohols, not carboxylic acids. However, when one considers that the toxicity of both of these compounds is largely attributed to membrane damage (Lennen et al., 2011, Jarboe et al., 2013, Reyes et al., 2013, Royce et al., 2013, Royce et al., 2014 and Zu et al., 2014), this result is consistent with our conclusion that the evolved strain has acquired a membrane with increased resistance to the damage caused by these compounds.

We were pleased to see that this evolved strain had increased production ability relative to the parent strain (Fig. 2). Though the titers achieved by this strain were lower than values

reported for other strains (Tee et al., 2014), it should be noted that only the most basic metabolic engineering strategies have been applied to this strain to enable carboxylic acid production, including abrogation of fatty acid uptake and acetate production and provision of an appropriate thioesterase. Other proven strategies, such as increased expression of fabZ (Ranganathan et al., 2012), and characterization in pH-controlled fermenters have not been applied.

We quantified the membrane of the evolved strain in regards to its fluidity, integrity (leakage) and composition (Fig. 3). Each of these metrics showed a significant difference from the parent strain. Most intriguingly, the evolved strain seems to have a more dynamic membrane (Table 3). This suggests that a change has occurred in the regulation of membrane composition. It should be noted that we have not proven that these changes in the membrane are the cause of the increased tolerance and increased carboxylic acid production. Other changes to the membrane, or other changes not explored here, may be of comparable or increased importance than the results described here. Other recent publications have highlighted the importance of the membrane when evolving strains for increased tolerance (Reyes et al., 2013 and Caspeta et al., 2014). Thus, it is increasingly apparent that metabolic engineering strategies for rationally strengthening the microbial membrane are needed.

Our production experiments were performed at 30 °C, and at this temperature the evolved strain has a higher S:U ratio than the parent strain (Fig. 3C). However, this trend is reversed at 42 °C, raising the interesting question of whether or not the evolved strain would still produce more carboxylic acids than the parent strain if evaluated at 42 °C. We have previously observed an antagonistic relationship between C8 adaptation and heat shock at 42 °C. MG1655 adapted to 30 mM C8 for 3 h has significantly more membrane leakage than heat shocked cells that were not adapted to C8 (Royce and Jarboe, unpublished data). Ongoing analysis being performed by

our group aims to identify the mutations that enable the evolved phenotype and, more importantly, understanding of how they support the evolved phenotype. The work described here demonstrates that (a) it is possible to increase carboxylic acid production via an increase in tolerance, (b) the strain with increased tolerance has altered membrane properties and (c) acquisition of tolerance to octanoic acid enables increased tolerance to the membrane-damaging biofuels of n-butanol and isobutanol.

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Tables

Table 1. Strains and plasmids.

Strain	**Genotype**	**References**
ML115 “parent”	MG1655 *fadD poxB ackA-pta*: cmR	(Zhang et al., 2012)
LAR1 “evolved”	ML115 evolved for C8 tolerance	This work
Plasmid		
pJMY-EEI82564	pTrc-EEI82564 thioesterase from *Anaerococcus tetradius* (Jing et al., 2011)	This work
pJTD1	pBad24-TorA-GFPmut3*	(Thomas et al., 2001)

Table 2. Characterization of evolved isolates in closed fermentation vessels with MOPS 2.0 wt% dextrose and 10 mM C8 at 37° C, 150 rpm, pH of 7.0. The parent strain had a specific growth rate of 0.28 h^{-1}.

Strain number (s)	**Specific growth rate (h^{-1})**
Mixed cultures	
1–5	0.65
6–10	0.64
11–15	0.54
16–20	0.64
21–26	0.63
Pure cultures	
1	0.6
2	0.67
3	0.67
4	*n.d.*
5 (LAR1)	0.69

Table 3. Mol% of various lipids and lipid groups in the cell membrane for parent strain ML115 and evolved strain LAR1 grown to mid-log in MOPS 2.0% dextrose at 37° C and an initial pH of 7.0 and then adapted to the indicated stressor for 3 h. Each value is an average and standard deviation of three biological replicates, except the evolved strain at 30° C, where n=2. Total C16 includes C16:1, C16:0 and C17cyc. Total C18 includes C18:1, C18:0 and C19cyc.
Bolded values indicate lipids whose relative content was significantly ($P \leq 0.05$) different between the two strains in the same condition.
*Indicates lipids whose relative content significantly ($P \leq 0.05$) differed in the same strain relative to the control condition.
Values in parentheses indicate the fold change relative to the same strain in the control condition. This is only shown for lipids whose relative abundance was significantly different from the control condition.

	C12:0	C14:0	C16:1	C16:0	C17cyc	C18:1	C18:0	C19cyc	Total C16	Total C18
Control										
parent	4±2	7±2	**11.6±0.8**	**45±2**	17±1	13.2±0.8	1.10±0.06	**1.9±0.1**	73±4	**16.2±0.8**
evolved	0.30±0.03	3.9±0.2	**8.7±0.1**	**48.7±0.1**	19.4±0.1	14.4±0.2	1.3±0.1	**3.3±0.1**	76.8±0.2	**19.0±0.2**
30 mM C8 at pH 7.0 (4.3 g/L)										
parent	**2.8±0.3**	**5.2±0.4**	**20.1±0.5*** (1.7±0.1)	**43.5±0.4**	8.4±0.4* (-1.9±0.2)	**16±1*** (1.2±0.1)	2.3±0.3* (2.0±0.3)	**1.4±0.1*** (-1.3±0.1)	72±1	**20±1*** (1.2±0.1)
evolved	**0.40±0.04*** (1.3±0.2)	**2.90±0.06*** (-1.36±0.06)	**18.8±0.2*** (2.15±0.04)	**46.6±0.1*** (-1.04±0.00)	8.6±0.7* (-2.2±0.2)	**19±1*** (1.35±0.07)	1.9±0.2* (1.4±0.2)	**1.9±0.2*** (-1.7±0.2)	74±1* (-1.04±0.01)	**23±1*** (1.22±0.05)
430 mM Ethanol (20 g/L)										
parent	**2.2±0.4**	**6.0±0.4**	**16.1±0.1*** (1.39±0.09)	**43.6±0.5**	**14.2±0.2*** (-1.1±0.1)	**15.6±0.1*** (1.18±0.07)	**1.1±0.1**	**1.20±0.03*** (-1.5±0.1)	**74.0±0.7**	**17.9±0.1*** (1.11±0.06)
evolved	**0.40±0.06**	**3.9±0.3**	**11.0±0.2*** (1.26±0.02)	**46.7±0.3*** (-1.04±0.01)	**17.6±0.4*** (-1.10±0.02)	**16.8±0.2*** (1.17±0.02)	**1.40±0.08**	**2.20±0.03*** (-1.50±0.04)	**75.4±0.2*** (-1.02±0.00)	**20.4±0.2*** (1.07±0.02)
30^{0}C										
parent	3.2±0.9	6.6±0.9	**17±1*** (1.4±0.2)	42.6±0.9	12.7±0.7* (-1.3±0.1)	**15.3±0.8*** (1.16±0.09)	**1.4±0.7**	1.5±0.6	**72±2**	**18±2**
evolved	2.5±0.6* (8±2)	6.7±0.4* (1.7±0.1)	**11.1±0.3*** (1.27±0.04)	43±1* (-1.13±0.03)	12.4±0.2* (-1.57±0.03)	**17.9±0.3*** (1.25±0.03)	**4.1±0.1*** (3.0±0.3)	2.4±0.2* (-1.4±0.1)	**66±1*** (-1.16±0.02)	**24.4±0.0*** (1.29±0.02)
42^{0}C										
parent	**4±1**	**8.9±0.9**	**14.6±0.0*** (1.26±0.08)	**45±1**	**12.3±0.2*** (-1.3±0.1)	**11.5±0.5*** (-1.1±0.1)	1.3±0.2	**1.40±0.06*** (-1.4±0.1)	**73±2**	**14.3±0.4*** (-1.14±0.07)
evolved	**0.5±0.1*** (1.6±0.4)	**5.3±0.2*** (1.34±0.08)	**12.7±0.2*** (1.46±0.03)	**49.7±0.0*** (1.02±0.00)	**14.0±0.1*** (-1.38±0.02)	**14.1±0.1**	1.6±0.2	**2.00±0.02*** (-1.60±0.04)	**76.4±0.3**	**17.8±0.2*** (-1.07±0.02)

Figures

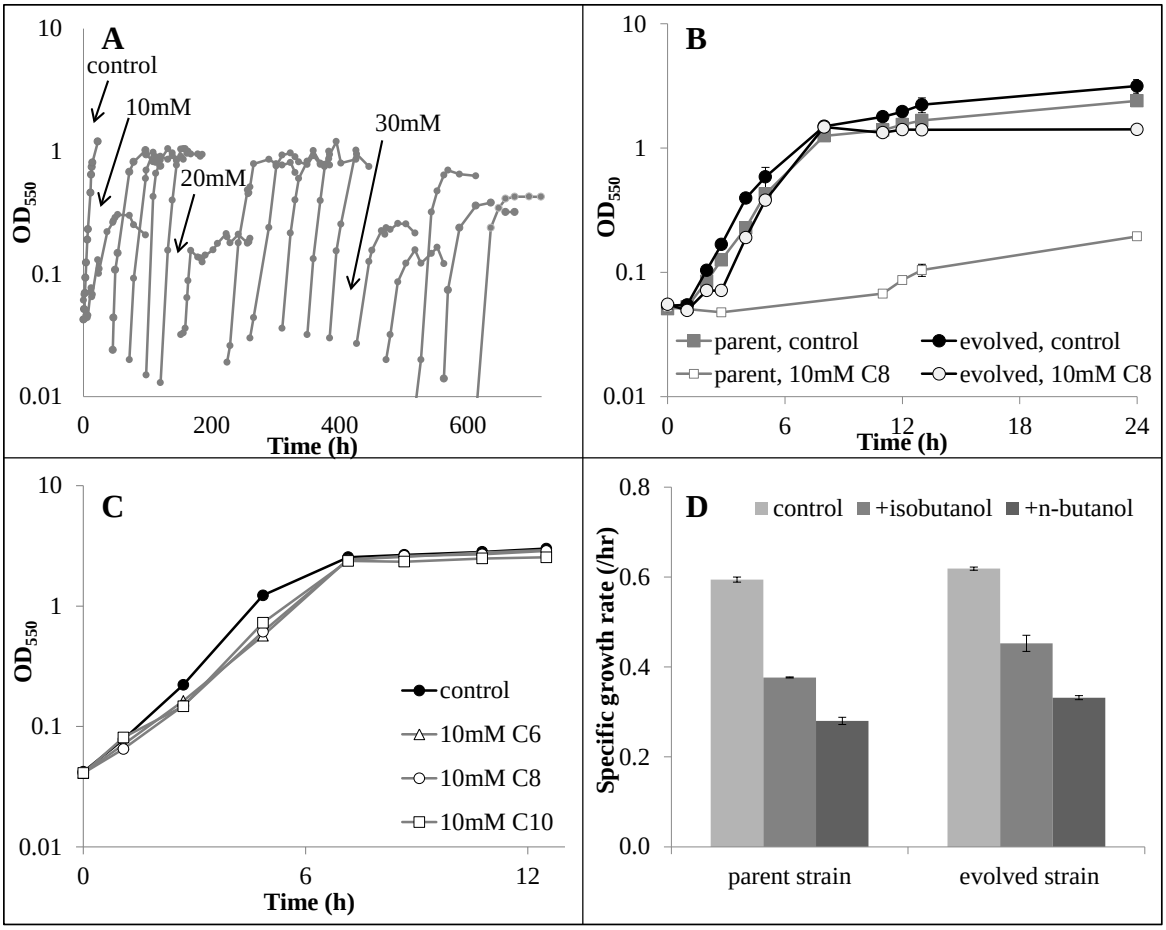

Figure 1. Development and characterization of the evolved strain. All experiments were performed at 37 °C in MOPS 2.0 wt% dextrose.
(A) Development of evolved strain LAR1 by serial dilution of parent strain ML115 in closed fermentation vessels at pH 7.0, 150 rpm. Arrows indicate the first transfer corresponding to each increase in octanoic acid concentration. (B) Evolved strain LAR1 has increased tolerance to exogenously supplied C8 relative to its parent ML115. (C) Evolved strain LAR1 shows similar growth in the presence of 10 mM C6, C8 or C10. (B and C) Strains were characterized in closed fermentation vessels at pH 7.0, 150 rpm. Data is the average of three biological replicates, with error bars indicating one standard deviation. (D) Evolved strain LAR1 has increased tolerance of 0.6% (v/v) isobutanol and n-butanol. Strains were grown in shake flasks at 200 rpm with an initial pH of 7.0. Values are the average of three biological replicates with error bars indicating one standard deviation. P values for parent strain vs evolved strain: control, 0.0032; isobutanol, 0.0018; n-butanol, 0.054 (t-test).

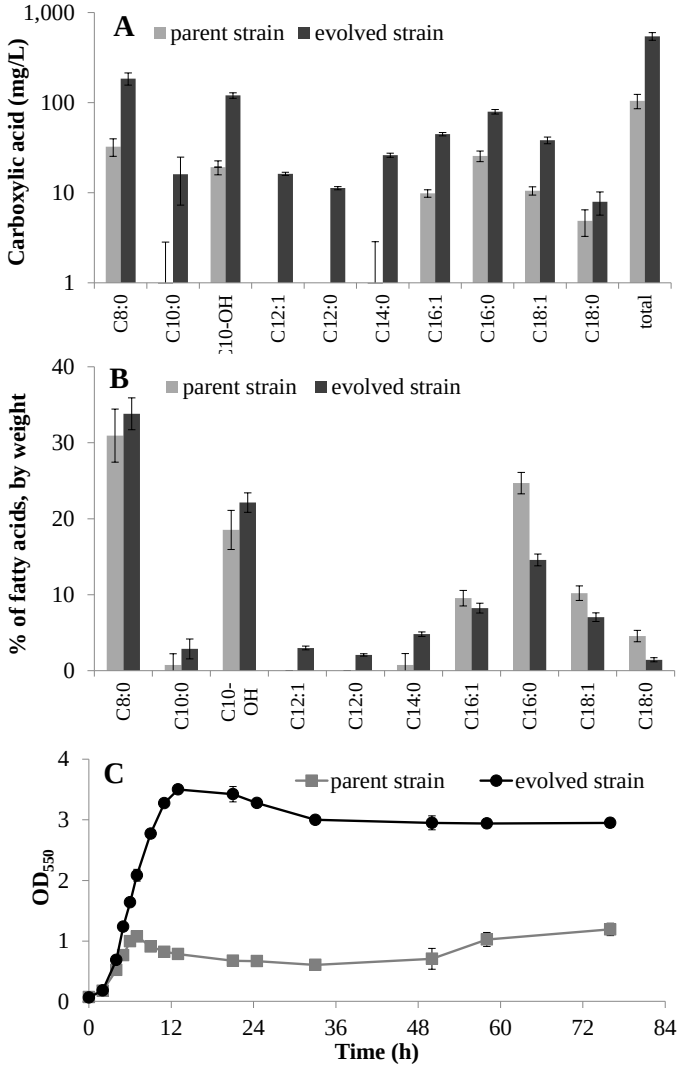

Figure 2. Evolved strain LAR1 shows increased carboxylic acid production relative to parent strain ML115. Both strains contain plasmid pJMY-EEI82564 encoding the *A. tetradius* thioesterase.

Strains were grown for 72 h in LB with 1.5% glucose at 30 °C, 200 rpm with 100 mg/L ampicillin and 1.0 mM IPTG. Values are the average of four biological replicates, with error bars indicating one standard deviation. (A) Carboxylic acid concentrations, all except C18:0 have a *P*-value of <0.05 using two-tailed *t*-test. (B) Relative distribution, by weight. C10–OH, C12:1, C12:0, C14:0, C16:0, C18:1, C18:0 have $P<0.05$. (C) Growth.

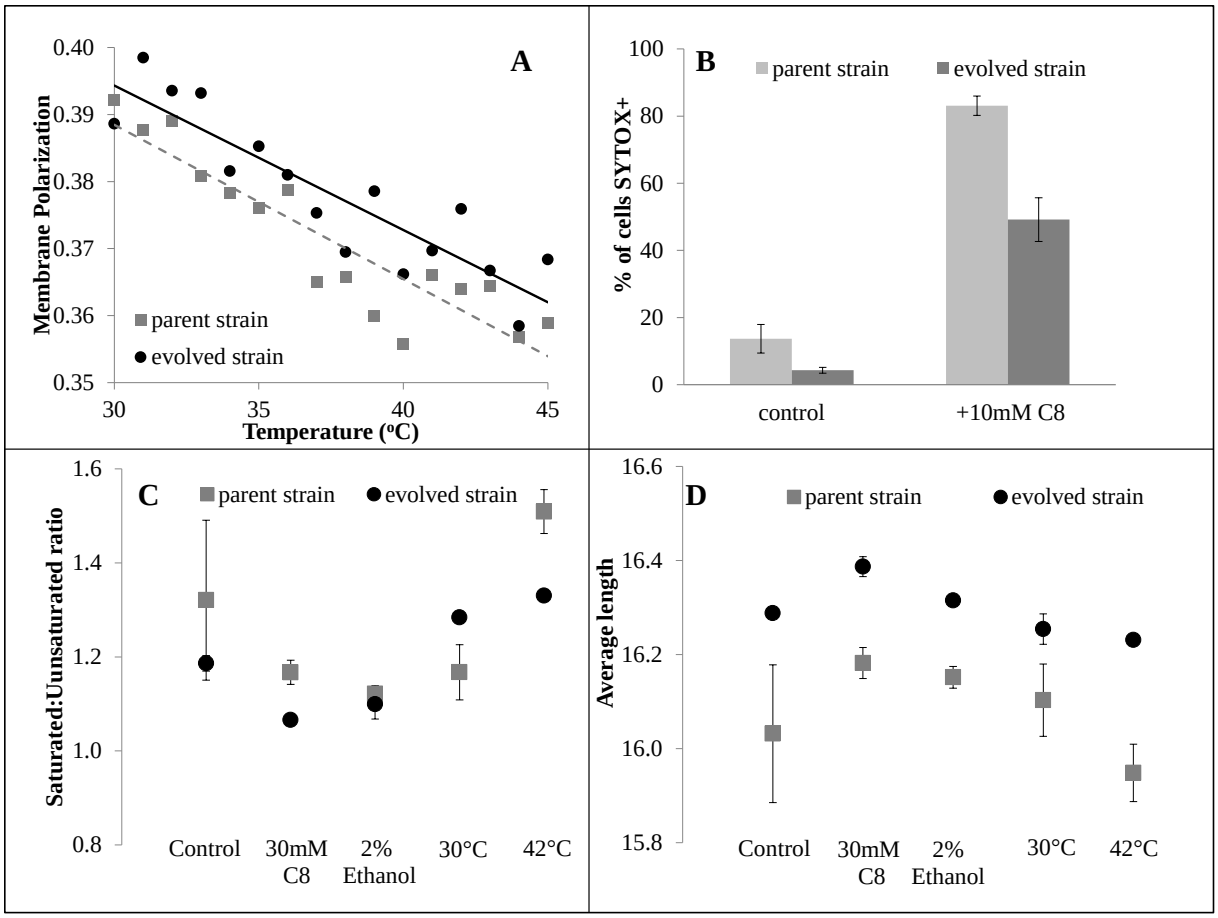

Figure 3. Evolved strain LAR1 has altered membrane characteristics relative to parent strain ML115.
(A) LAR1 has a more polarized membrane than ML115. Cells were grown in MOPS 2.0% dextrose at 37 °C and concentrated in PBS pH 7.0 with DPH. P=2×10−4 by paired two-tailed t-test. (B) LAR1 has increased membrane resistance to exogenously supplied C8 relative to ML115. Cells were grown in MOPS with 2.0% dextrose to OD550 ~1, challenged with C8 at pH 7.0 and then treated with SYTOX and analyzed by flow cytometry. Values are the average of three biological replicates with error bars indicating one standard deviation. P values for parent strain vs evolved strain: control, 0.056; +C8, 0.0052. (C, D) Membrane saturated:unsaturated ratio and average lipid length. Cells were grown to mid-log in MOPS 2.0% dextrose at 37 °C, harvested, resuspended in fresh media and then incubated in the indicated condition for 3 h. The S:U ratios are significantly different ($P<0.05$) between strains for 30 mM C8 and 42 °C. The average lipid length was significantly different ($P<0.05$) between strains for all conditions. Each value is an average and standard deviation of three biological replicates, except the evolved strain at 30 °C, where n=2.

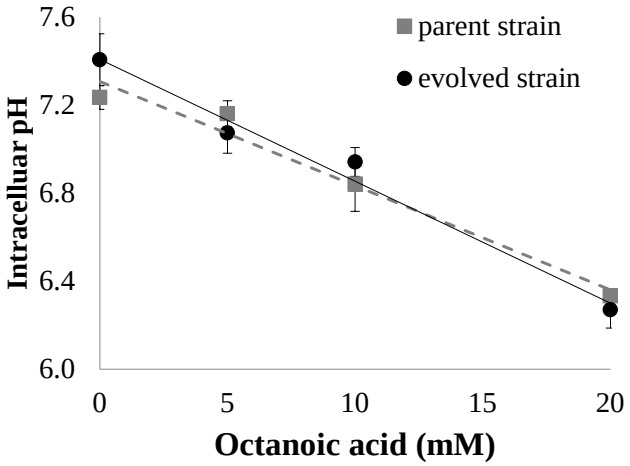

Figure 4. Evolved strain LAR1 has comparable intracellular pH to parent strain ML115 in response to exogenous challenge with C8.
Strains encoding pH-sensitive GFP via pJTD1 were grown in LB at 37 °C with 100 mg/L ampicillin and 200 μM l-arabinose, concentrated and then challenged with C8 at pH 7.0. Paired t-test showed no significant difference between the two strains.

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CHAPTER 4. GENOME-LEVEL REVERSE ENGINEERING OF *ESCHERICHIA COLI* EVOLVED FOR INCREASED SHORT-CHAIN FATTY ACID TOLERANCE AND PRODUCTION

Draft of a research paper to be submitted to *Metabolic Engineering*

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Introduction

Fatty acids are of great importance in the industrial field due to their wide applications as multifunctional precursors to produce various fuels, chemicals, and textile fibers (1–4). So far, the production of industrial fatty acids relies heavily on a nonrenewable and unsustainable resource, petroleum (2, 4), which can lead to severe environmental, political, and economic consequences (5). Therefore, it is necessary to develop new pathways to produce fatty acids using renewable and sustainable carbon feedstocks. In this respect, biocatalysts are attractive and promising. They have already been broadly applied to biorenewable industries for the production of various chemicals, such as ethanol, glycerol, 1, 3-propanediol, and lactic acid (6). Moreover, it is potentially feasible for researchers to engineer organisms to obtain the biocatalysts with significant ability to produce fatty acids since organisms can naturally synthesize fatty acids with 12-18 carbons, the primary components of the microbial cell membrane (6). Although, big progress has been made for increasing the production of fatty acids by biocatalysts (7–10), inhibition of fatty acid has been reported as a limiting factor of the bio-production.

Researchers have found that fatty acids are toxic to the microbes at concentration below the desired yield and titer (1, 11, 12), the same occurs with other desired biofuels and biorenewable chemicals (13–15). Toxicity is a major obstacle for boosting the yield and titer of fatty acids (1). The mechanism of the toxicity of fatty acid to *Escherichia coli* has been studied. It was reported that fatty acids can reduce the cell viability by damaging the cell membrane and decreasing intracellular pH (7, 11, 12). Several groups have tried to overcome this by different approaches. One strategy was to restore the cell membrane to improve cell viability by expressing one gene *fabR* and two different acyl-ACP thioesterase genes in *E. coli*. However, the engineered *E. coli* did not show improved fatty acid productivity (11, 16). Another one was to delete the acyl-ACP synthase (*aas*) gene in *E. coli*, which resulted a decreased percentage of medium-chain fatty acids in the membrane, increased tolerance to medium-chain fatty acids, and a slightly improved yield of fatty acids (17). Our group currently focuses on increasing the tolerance of *E. coli* to short-chain fatty acids (SCFAs) *via* adaptive laboratory evolution. The evolved strain has increased SCFAs tolerance and improved fatty acid production titer (7).

Reverse engineering aims to identify the crucial mutations that enable evolved strains to obtain the evolved phenotype and to understand why these mutations are beneficial. It is a powerful tool to elucidate the mechanisms behind the evolution experiments, these mechanisms can be used as design strategies for improving tolerance and production of biocatalyst. Identifying and understanding the key mutations that support the evolved phenotype requires knowledge of what mutations occurred during the evolutionary engineering. Whole-genome sequencing is very important for finding the critical mutations (18), and transcriptome analysis and metabolic flux analysis have been proven quite useful for revealing the underlying mechanisms of the mutations (19, 20). Once the important mutations are found, the next steps are

to explore which mutations promote fitness, and understand the mechanisms of inhibition and the functions of poorly-characterized enzymes and pathways. For instance, in an isobutanol tolerance study, five mutations were identified primarily responsible for the increased isobutanol tolerance, and glucosamine-6-phesphate was identified as an important metabolite for isobutanol tolerance in *E. coli* (21). Increasing furfural tolerance was achieved by silencing the NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in *E. coli* (20). The new glucose uptake system and mechanism of increased ATP level in the evolved strain has been well studied and were found to be essential for improving succinate production in *E. coli* (22).

In this work, we investigated the evolved *E. coli* strains by employing reverse engineering to understand the mechanisms of the increased short-chain fatty acid tolerance and production. Four mutations were verified in evolved strains. We further studied each mutation contribution on short-chain fatty acid tolerance, fatty acid production, and cell membrane properties. All mutations were found to be important and they all somewhat contribute to the changed phenotypic of evolved strains.

Materials and Methods

Strains, plasmids and bacterial cultivation

All strains and plasmids used in this study are listed in Table 1 and Table 2. *E. coli* DH5α strain was used as a cloning strain, while the parent strain ML115 and the evolved strain LAR1 were used for genome modification. Overnight seeds cultures were grown in 250 mL flasks with 25 mL of MOPS minimal media with 2 wt% dextrose at pH 7±0.05, 37 ºC, and 250 rpm. The overnight cultures were diluted to an optical density at 550 nm (OD_{550}) of 0.05 for the octanoic acid tolerance test, or diluted to an OD_{550} of 0.1 for testing membrane leakage, membrane fluidity, cell hydrophobicity, and cell membrane composition. *E. coli* transformants

were grown in media at 37 ºC, or 30 ºC. Chloramphenicol (35 mg/L), ampicillin (100 mg/L), kanamycin (50 mg/L), or spectinomycin (50 mg/L) was added as needed.

Whole-genome sequencing & verification of mutant genes

The genomic DNA of ML1155, LAR1 and LAR2 was purified using the Qiagen Blood and Tissue kit. The Illumina Genome Analyzer II platform for high throughput sequencing was used for whole genome sequencing with 77 bp (base pair) paired-end reads as described (18). The softwares and algorithms used for genome assembly and obtaining the set of predicted mutations were previously described (18). Breseq version 0.26.1, a pipeline for finding mutations in microbial genomes, was used to analyze the short read data (23). Breseq aligns reads to a reference genome, in our case wild-type *E. coli* K-12 MG1655 U00096.2 (24), using the Bowtie2 alignment algorithm (25). Genomes of the evolved and parent strains were aligned to the wild-type genome and variations in the evolved strain that were not present in the parental were selected as mutations of interest to be verified with PCR and Sanger sequencing.

The whole potential mutated gene sequence with additional 500 base pairs (bp) upstream and downstream sequences were verified in order to verify mutations with more confidence. All primers were designed by primer3 online software and synthesized by Integrated DNA Technologies (IDT). The size of PCR products was first checked on a 1% TAE agarose gel with 1Kb plus DNA ladder (Invitrogen Life Science). Then PCR products were purified by QIAquick PCR purification kits (Qiagen) and submitted to Iowa State University DNA facility for DNA sequencing analysis. The sequencing result were compared with the MG1655 genome using the online Standard Nucleotide BLAST software. Verified mutations were double checked by repeating all steps above using the genome of evolved strain and parent strain separately as templates.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-FRLP was employed to determine the chronological order of mutations during adaptive evolution. Cells cultures saved before every transfer during adaptive evolution were used as template DNA for PCR. For the *rpoc* (A1256C) mutation, the 660 bp DNA fragment which includes the mutant point was amplified by PCR with the primers rpoCCF, rpoCCR and DreamTaq Green PCR master mix 2X (Thermo Fisher Scientific). The PCR products were purified by DNA Clean & Concentration kit (Zymo). Approximately 10 µl of purified PCR products were digested with restriction enzyme BsaJI (New England Biolabs) according to the manufacturer's instruction. The restriction fragments were separated on a 1% TAE agarose gel with 1Kb plus DNA ladder. The pair of primers basRCF and basRCR, and restriction enzyme SfcI were used for digesting *basR* PCR product and identify *basR* mutation. The basSCF and basSCR primers, and restriction enzyme FatI were used for identifying *basS* mutation. For the *waaG* mutation, waaGCF and waaGCR primers were needed.

Genomic manipulations

All genomic manipulations were carried out using either lambda red recombinase system (26) or CRISP-cas9 system (27). For the lambda red recombinase system, *E. coli* strains were first transformed by electroporation to harvest the pKD46 plasmid, and then the lambda red recombinases could be induced by adding L-arabinose (2 mM). The kanamycin resistance cassette was amplified from plasmid pKD4 by PCR using primers with flanking homologous regions for the target gene. The rpoC(1256A)+kan, and rpoC(1256C)+kan cassettes were synthesized by GenScript company. The purified PCR products were transformed into the electro-competent *E. coli* cells harboring pKD46, and the lambda red recombinase system was induced. The resulting kanamycin resistant colonies were screened for the successful gene

replacement by the PCR amplification, restriction enzyme digestion, and DNA sequencing. The scarless CRISPR-Cas9 approach was also applied to perform gene editing (27).

Octanoic acid tolerance test

Octanoic acid (C8) tolerance was determined by measuring OD_{550} every hour starring from two hours after inoculating, until growth get into stationary phase, twenty-four hours OD_{550} was recorded as a metric of C8 tolerance test. Overnight seed cultures were inoculated into 250 mL baffled flasks with 25 mL MOPS with 2.0 wt% dextrose with 10 mM octanoic acid (1.44 g/L), at pH 7±0.05, 37 ºC, and 200 rpm. The control groups were growing in the same culture without exogenous octanoic acid.

Fermentation for fatty acid production

The strains transformed with the pJMY-EEI82564 plasmid were grown on LB plates with 100 mg/L ampicillin and incubated at 30 ºC overnight. Individual colonies were cultured in 250 mL flasks with 10 mL LB media and 100 mg/L ampicillin, at 30 ºC on a rotary shaker at 250 rpm overnight. Seed cultures were inoculated into 250 mL baffled flasks containing 50 mL of LB media with 1.5 wt% dextrose, 100 mg/L ampicillin, and 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The initial OD_{550} was approximately 0.1. The flasks were incubated in a rotary shaker at 250 rpm and 30 ˚C. At 6 h, 12 h, 24 h, 48 h, and 72 h, culture samples were saved for testing fatty acid titer.

Determination of fatty acid titers

Fatty acid production was quantified by fatty acid extraction and further derivatization from samples containing both media and cells. The FAMEs measured via an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectroscope (GC-MS) at the ISU W.M. Keck Metabolomics Research Laboratory, as previously described (28). Briefly, 1 mL culture was

transferred into a 2 mL microcentrifuge tube, 125 µL 10% NaCl (w/v), 125 µL acetic acid, 20 µL internal standard (1 µg/ µL C7, C11, C15 in ethanol), 500 µL ethyl acetate was added sequentially. The mixture was vortexed 30 s and centrifuged at 16,000×g for 10min. Then, 250 µL of the top layer (contain free fatty acid) was transferred into a glass tube. For the fatty acid derivatization part, 2.25 mL 30:1 EtOH: 37% HCl (v/v) was added into the glass tube from fatty acid extraction part, incubated at 55 ºC for 1 hour, then cooled to room temperature. After this, 1.25 mL ddH_2O and 1.25 ml hexane were added, followed by vortexing and centrifugation at 2,000×g for 2 min. The top hexane layer was then analyzed by GC-MS using the following programs: the initial temperature was set at 50 °C, holding for 1 min, with the following temperature ramp: 20 ºC/min to 140 ºC, 4 ºC/min to 220 ºC, and 5 ºC/min to 280 ºC with 1 ml/min helium carrier gas. The relative retention factor of C7/C11/C15 was used to adjust the relative amounts of the individual fatty acids analyzed. The Enhanced Data Analysis (Agilent Technologies) and NIST 17 Mass Spectral Library software were used for peak identification.

Extracellular polymeric substance (EPS) extraction and quantification

The total extracellular protein and polysaccharide was determined by an extraction method (29). Briefly, *E. coli* cells were incubated on LB agar plates overnight at 37 ºC to obtain the total amount cell within the range of 2×10^{11} to 4×10^{11} cells. Then, cells were suspended in 30 mL 0.85 wt% NaCl solution. Cell concentration was measured by the flow cytometry with CountBright absolute counting beads (ThermoFisher Scientific), at ISU Flow Cytometry Facility. The cell suspension was centrifuged at 16,300×g, at 4 ºC for 30 min, the supernatant was filtered through a 0.45 µm filter and 90 mL of ice-cold 100% ethanol were added. The mixture was incubated at -20 ºC for 24 h. Then, the EPS pellet was harvested by centrifuging at 16,300×g for 30 min at 4 ºC and air-dried in a fume hood. The EPS pellet was resuspended in 20 mL DI water

and ready for the protein and sugar analysis. The Lowry method (30) was used to measure EPS protein, which is a spectrometric method based on measurement at a wavelength of 500 nm by using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as the standard. The phenol-sulfuric acid method was used to analyze EPS sugar, which is based on measurement at a wavelength of 488 nm using xanthan gum as the standard (31).

Membrane characterization

Membrane permeability: The seed culture was inoculated into 250 mL baffled flasks with 25 mL MOPS media with 2 wt% dextrose, at 250 rpm and 37˚C. The cells were harvested at mid-log phase (OD_{550}~1) followed by centrifugation at 4,500×g and 22 ºC for 10 min. Then, cells were treated with PBS with 10 mM octanoic acid at pH 7.0, incubated at 37 ºC for 1 hour. The control group treated with PBS without exogenous C8. Subsequently, cells were centrifuged at 4,500×g at 22 ºC for 10 min, washed twice with PBS, and resuspended in PBS at a final OD_{550}~1. Then, 100 µL resuspended cells were diluted with 900 µL PBS. Cells were stained with 1 µL of 5 mM SYTOX green in dimethyl sulfoxide (Invitrogen, Carlsbad, CA) (16). Finally, cells were analyzed by flow cytometric on the BD Biosciences FACSCanto II, at ISU Flow Cytometry. About 18,000 events were tested per sample, and each sample had three parallel groups.

Membrane fluidity: Membrane polarization was measured using 1, 6-diphenyl-1, 3, 5-hexatirene as previously described (12, 32). Briefly, cells were treated as described in membrane permeability part. Cell pellets were resuspended in PBS at a final OD_{550}~1. Then, transferred 500 µL resuspended cells into a 1.5 mL centrifuge tube containing 500 µL of 0.4 µM DPH in PBS. The mixture was vortexed, and incubated at 37 ºC in dark for 30 min. Then the treated cells were centrifuged (5,000×g, 5 min) and the cell pellets were resuspended in 500 µL PBS. One hundred

μL of this mixture was transferred into sterile black-bottom Nunclon delta surface 96-well plate with 4 replicates. The suspension of cells without DPH treatment was used as control. Membrane fluorescence polarization values were determined based on vertical and horizontal fluorescence readings, which was assessed by the BioTek Synergy 2 Multi-Mode microplate reader at ISU W.M. Keck Metabolomics Research Laboratory.

Cell surface hydrophobicity: The method of measuring cell surface hydrophobicity was based on the previously described procedure (33, 34). The mid-log cells were harvested and treated with MOPS with 2 wt% dextrose with or without 10 mM octanoic acid, at pH 7.0 and 37 ˚C with rotary shaking at 250 rpm for 1 hour. Then, cells were washed twice with PBS, and resuspended in PBS at a final OD_{550} ~ 0.6. Four mL cell suspension were added to a glass tube, and the OD_{550} was measured as OD_1. Then, 1.0 mL dodecane was added, and the mixture was vortexed using a multi-tube vortexer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 2500 rpm for 10 minutes to homogenize the aqueous and organic phases. The mixture were left to stand for 15 min to allow phase separation. The OD_{550} of the aqueous phase (OD_2) as measured. Partitioning of the bacteria suspension is calculated as:

Percent partitioning= $[(OD_1-OD_2)/OD_1]*100$

Membrane lipid composition: The mid-log cells were harvested and treated with MOPS with 2 wt% dextrose with or without 30 mM octanoic acid at pH 7.0, and incubated for 3 hours at 37 ºC. Then, cells were washed twice with cold sterilized water, and then resuspended in 6 mL methanol, transferred of 1.4 mL cells suspension into glass tubes with three replicates (35). Twenty μL of 1 μg/ μL C7, C11, C15 in methanol was added as internal standards. The mixtures were sonicated for three 30 s bursts, incubated at 70 ºC for 15 min, and cooled to room temperature. The cells were centrifuged at 4000×g for 5 min. The supernatant was transferred

into a new glass tube with 1.4 mL nanopure water, and the mixture was vortexed. 750 µL of chloroform was added to the cell pellet, followed by vortexing and shaking in a horizontal shaker at 150 rpm, 37 ºC for 5 min. The diluted supernatant was transferred back to the chloroform-treated pellet. The mixture was vortexed for 2 min, then centrifuged at 3,000×g for 5min. The bottom chloroform layer which contain free fatty acid was transferred to a new glass tube. All solvent removed with an N-Evap nitrogen tree evaporator. For the fatty acid derivatization part, 2 mL of 1N HCl in methanol was added to the dried samples, heated at 80 ºC for 30 min, then cooled to room temperature. Then, 2.0 mL 0.9 wt% NaCl and 1.0 mL hexane was added followed by vortexing for 2 min and centrifugation at 2,000×g for 2 min. The upper layer containing the hexane with FAMEs was transferred into a GC vial for analysis, using the method described above. The weight-average lipid length were calculated as previously described (12).

Results and Discussion

Verified mutations in evolved strains

To identify mutations in evolved strains LAR1 and LAR2, the genomic DNA of ML115, LAR1, and LAR2 was sequenced by using the Illumina platform. We used the short-readers aligner, Bowtie and Velvet, to mapping the short-sequenced reads to the reference genome, MG1655. Potential mutations including single nucleotide polymorphisms (SNPs), deletions, insertions, and duplications could be identified by mapping short-sequenced reads of evolved strains, parent strain to the reference genome. Each potential mutation was verified by PCR and Sanger Sequencing. In summary, three insertions, respectively, in *waaG* gene, *yeaJ* gene, and *yebB* gene were verified in the genome of parent strain ML115. LAR1 was verified to contain two single amino acid changes, one was H419P in RpoC, another was D28Y in BasR gene, and the insertion in *waaG* gene was removed during adaptive evolution (Table 3). LAR2 was verified

to contain the same single amino acid change in RpoC (H419P), a 9-amino acids deletion in BasS, and the insertion in *waaG* gene was also removed (Table 3).

The RpoC (H419P) mutation was identified in both evolved strains, which encodes the β' subunit of the RNA polymerase sigma 70 factor. The RNA polymerase sigma 70 factor interacts with sigma 70 promoter which controls expression of about 1000 genes in *E. coli* K-12 (36). Thus, this mutation in *rpoC* gene might widely affect the genes expression in the evolved strains. Different mutations in *rpoC* gene were found in other evolutionary studies of acid tolerance, and the mutated RpoC (V507L) contributed to increased acid-tolerant phenotype (37). It is highly possible the RpoC (H419P) mutation contributed to the increased C8 tolerance of the evolved strain. We also confirmed mutation in BasR (D28Y) in LAR1, which encodes the transcriptional regulator component of BasS/BasR system. The BasS-BasR two-component system is one of the two component signal transduction systems in *E. coli* which sense and respond to changes in environmental conditions (38). In an evolutionary study of n-butanol tolerance, overexpression of the *basS* gene increased n-butanol tolerance (14). Interestingly, nine amino acids deletion in BasS was verified in LAR2, which encodes sensory histidine kinase of the BasS/BasR system. The 768 base-pair insertions (InsB-5, InsA-5, and InsAB-5) in *waaG* gene in parent strain ML115 could stop the expression of WaaG and likely change the expression level of WaaP, WaaS, WaaS, WaaO, WaaJ, OmpR, and LrhA (Fig. 1). The WaaG is a lipopolysaccharide (LPS) glucosyltransferase I and adds the first glucose of the outer core of LPS (39). The deletion of *waaG* gene results in a truncated LPS core, loss of flagella pili (40), enhanced cell surface hydrophobicity, increased outer membrane permeability, and decreased ability of biofilm formation (41). The deletion of *waaGPBI* leads to a mucoid colony morphology (40), which is consistent with the morphological character of ML115 on LB plate (Fig. 2A). The TEM image of

ML115 showed cells have no flagella and pili (Fig. 2C). All verified mutations are of interest for further investigation of cell membrane properties.

Identification of the chronological order of mutations

Knowing the chronological order of mutations could provide a clear strategy to investigate each mutation and the synergistic interaction of these. The *waaG* gene has a 768 bp insertion sequence in the parent strain ML115, the *waaG* gene with insertion (1714 bp) and the restored *waaG* gene (946 bp) were identified. From Figure 3A, the fragment length of ML115 in lane 2 is 1714 bp, the fragment length of LAR1 in lane 17 is 946 bp. The cell population before the third transfer during adaptive evolution all had the restored *waaG* gene (Fig. 3A).

The *rpoC* mutation A1256C could not be identified by fragment length like *waaG* mutation. However, combining PCR and digestion reaction with the restriction enzyme BsaJI, the *rpoC* mutation could be identified (Fig. 4A). The size of PCR produces of wild-type and mutant *rpoC* were both 660 bp. The restriction enzyme BsaJI was able to digest the wild-type *rpoC* fragment into two pieces 427 bp and 233 bp. The mutant *rpoC* fragment was digested into three pieces 208 bp, 219 bp, and 233bp. The PCR-RFLP results showed the parent strain ML115 in lane 2 has two bands 427 bp and 233 bp, the evolved strain LAR1 in lane 17 has only one band around 200 bp. The cell population all had the mutant *rpoC*, before 13^{th} transfer during adaptive evolution (Fig. 3B).

The similar PCR and digestion reactions were able to design for *basS* and *basR* mutations (Fig. 4BC). However, according to the PCR-RFLP results, *basS* and *basR* mutations were not able to found in the cell population during adaptive evolution (Fig. 3CD). We hypothesize that *basS* and *basR* mutations showed up in the end of adaptive evolution, and the cells containing either *basS* mutation or *basR* mutation only were a small population. During adaptive evolution,

the chronological order of mutations occurred as follows: *waaG* mutation, *rpoC* mutation, and then *basS* or *basR* mutations.

Investigation of the contribution of mutations on C8 tolerance

Evolved strain LAR1 had significantly increased short-chain fatty acid tolerance phenotype relative to parent strain ML115 (7). In order to study the contribution of each mutation on C8 tolerance, we systematically constructed 12 strains, including strains with single mutation, double mutations, or triple mutations into the genome of parental strain ML115 (Table 1). Firstly, we followed the chronological order of mutations to study their contributions on C8 tolerance. The parent strain ML115 barely grew in MOPS with 2 wt% dextrose with 10 mM C8 (Fig. 5A). With the restored *waaG* gene, the ML115+waaGInD strain showed clearly increased C8 tolerance (Fig. 5B). However, with exogenous 10 mM C8 challenging, the specific growth rate of ML115+waaGInD strain was only 0.39 h^{-1} (Table 3), which was lower than the specific growth rate of the evolved strain LAR1 (0.56 h^{-1}). Then, the ML115+waaGInD+rpoC* strain showed further increased C8 tolerance relative to ML115+waaGInD (Fig. 5C). The specific growth rate of ML115+waaGInD+rpoC* strain was able to achieve 0.55h^{-1}, with exogenous 10 mM C8 challenging (Table 3). The reconstructed strain with restored *waaG* gene and mutant *rpoC* gene had the same level of increased C8 tolerance with the evolved strain LAR1 (Fig. 5 C-E). In contrast, the reconstructed strain ML115+waaGInD+rpoC*+basR* showed no further increased C8 tolerance relative to the ML115+waaGInD+rpoC* (Fig. 5D). These results determine that the restored *waaG* gene and mutant *rpoC* gene are the two mutations mainly contributed on increased C8 tolerance of evolved strain LAR1.

Then, we looked into other reconstructed strains to deepen the understanding of the contribution of mutations on C8 tolerance. The reconstructed strains YC002, YC003, YC004,

YC008, YC009 with disturbed *waaG* gene were not able to grow in MOPS media with 2 wt% dextrose with 10 mM C8 (Table 3). The reconstructed strains YC006, YC007, YC011 with wild-type *rpoC* gene had specific growth rate lower than 0.4, with exogenous 10 mM C8 challenge. These results demonstrated that the chronological order of mutations was very important for reverse engineering the evolved strain. Following the order to investigate mutations could save more time when constructing strains and allowed us to have a better understanding of the evolved strains and the synergistic interactions of their mutations.

The effect of identified mutations on fatty acid titer

Since the reconstructed stains ML115+waaGInD and ML115+waaGInD+rpoC* demonstrated increased C8 tolerance when challenged with 10 mM C8, we tested their contribution to the increased short chain fatty acid production in the LAR1 strain. All strains contain plasmid pJMY-EEI82564 encoding the *A. tetradius* thioesterase (TE10), which primarily produces octanoic acid (42). The parent strain ML115 only produced about 80 mg/L fatty acid after 72 hours (Fig. 6A). The fatty acid titer of ML115+waaGInD was approximately 188 mg/L (Fig. 6A). The ML115+waaGInD+rpoC* strain was able to produce 782 mg/L fatty acid, as much as the evolved strain, which had a 9-fold increase in fatty acid production relative to parent strain (Fig. 6A). The OD_{550} value for the ML115+waaGInD+rpoC* and LAR1 had more than 4.4-fold increase relative to the parent strain over the 72 hours fermentation (Fig. 6B). Thus, the 9-fold increase in fatty acid production cannot solely be attributed to increased biocatalyst abundance.

Parental strain ML115 had dramatic high content of EPS polysaccharides

The *waaG* gene encodes to the lipopolysaccharide (LPS) glucosyltransferase I, which adds the first glucose of the outer core of LPS (39). We did extraction and quantification of the

two major components of the extracellular polymeric substance (EPS); polysaccharides and proteins, to determine if the disturbed *waaG* gene had an effect on the content of EPS. The cells of parent strain ML115 with disturbed *waaG* gene had dramatic high content of polysaccharides, about 2.8 µg/ (10^8 cells), which was at least 12-fold higher than the strains with restored *waaG* gene (Fig. 7). The parent strain ML115 with disturbed *waaG* gene should only have the inner core of LPS while the strains with restored *waaG* gene should have the complete LPS. These results indicate that the cells of parent strain ML115 have a large increase amount of LPS with only inner core part relative to strains with restored *waaG* gene. We have hypothesized that the strain with disturbed *waaG* gene has only inner core of LPS and this might lead to these mutant cells to overexpress all additional genes related to LPS synthesis as a defense mechanism to overcome the lack of inner core.

Identified mutations had an effect on the cell membrane properties

It is known that cell membrane characterization is very important in the study of increasing microbial tolerance and production of biorenewable fuels and chemicals (8, 10, 16, 42–44). From previous studies, the cell membrane damage is the main mechanism of short-chain fatty acid toxicity (7, 11, 12). The evolved strain LAR1 had improved membrane integrity relative to parent strain ML115 is reported in literature (7). Here, we did cell membrane characterization of ML115+waaGInD, ML115+rpoC*, ML115+basS* and ML115+basR* strains, to understand how each mutation was able to alter the cell membrane properties. In order to study the synergistic interaction of mutations on cell membrane, we also did the same cell membrane studies to ML115+waaGInD, ML115+waaGInD+rpoC* and ML115+waaGInD+rpoC*+basR* strains.

We used SYTOX Green to assess membrane permeability, where permeability to this compound indicates a damaged membrane. Then SYTOX-permeable and SYTOX-impermeable cells population could be separated and quantified by flow cytometry. This analysis showed 88% of the parent strain ML115 population became SYTOX-permeable in the 0 mM C8 control condition and 10 mM C8 treatment condition (Fig. 8). In accordance to our tolerance results, the ML115+waaGInD, ML115+waaGInD+rpoC*, ML115+waaGInD+rpoC*+basR* and LAR1 strains with restored *waaG* gene, had less than 20% of SYTOX-permeable population, (Fig. 8A). These results determined parent strain ML115 has a serious membrane leakage problem and that by restoring the *waaG* gene this particular condition could be solved. This is consistent with previous reports demonstrating that deletion of *waaG* gene in *E. coli* increased outer membrane permeability (41). We also noticed that with only the *rpoC* mutation, the strain still had high percentage of SYTOX-permeable population (Fig. 8B). Interestingly, the SYTOX-permeable populations of ML115+basS* and ML115+basR* strains were lower than 65.4 %, only when the cells were challenged with exogenous 10 mM C8. These results indicated that strains with either *basS* or *basR* mutations had increased ability to adjust the 10 mM C8 challenge condition by preventing cell membrane leakage problem.

The evolved strain LAR1 had significantly higher membrane polarization than the parent strain in the temperature range from 30-45 ºC (7), which corresponds to lower membrane fluidity and higher membrane rigidity. Here, we tested cell membrane polarization of the strains with and without 10 mM C8 exogenous challenge. In accordance to previous reports, the evolved strain LAR1 had significantly higher membrane polarization relative to parent strain ML115 in both conditions (Fig. 9A). This increased membrane polarization was likely mainly caused by the synergistic interaction of the three identified mutations under the control condition (Fig. 9A). In

contrast, none of the constructed strains with single mutations was able to recreate the same level of increased membrane polarization exhibited by the evolved strain LAR1 (Fig. 9B).

In order to have a better understanding of the changes in cell membrane, we quantified the cell membrane lipid composition of the reconstructed strains, cells were treated with or without 30 mM C8 challenge. The evolved strain had significantly increased weight-average lipid length relative to parent strain (Fig. 10A), which is consistent with our previous report (7). Interestingly, the ML115+rpoC* strain had the same weight-average lipid length as LAR1 in both conditions (Fig. 10B). We also noticed that the parental strain with restored *waaG* gene had significantly increased weight-average lipid length relative to parent strain (Fig. 10B). The *basS* and *basR* mutations had no consistent effect on weight-average lipid length in both conditions. Increasing weight-average lipid length could be a strategy for potentially enhancing membrane integrity.

From previous report, the deletion of *waaG* gene resulted in enhanced cell surface hydrophobicity (41). Thus, we measured the surface hydrophobicity of the reconstructed strains to study whether these mutations lead to changes in cell surface hydrophobicity. In both control and 10 mM C8 conditions, the parent strain ML115 had very low cell surface hydrophobicity (1%) (Fig. 11A). The cell surface hydrophobicity of evolved strain was significant higher than ML115 in control condition and 10 mM C8 condition (Fig. 11A). This large increase in cell surface hydrophobicity of evolved strain LAR1 was likely caused by the synergistic interaction of the identified three mutations (Fig. 11A). Especially, in the 10 mM C8 condition, none of the parent stains with single mutations had that large increase in cell surface hydrophobicity (Fig. 11B).

The evolved strain had significant differences compared to the parent strain in all cell membrane characterization parameters we studied. We were pleased to see all mutations contributed to at least one cell membrane property. Cell membrane leakage could be prevented by restoring the *waaG* gene and this was the most helpful mutation for membrane integrity. We also noticed that the *basS* and *basR* mutations can partially mitigate the membrane leakage problem in the 10 mM C8 condition. The increased membrane polarization and cell surface hydrophobicity were caused by the synergistic interaction of *waaG*, *rpoC* and *basR* mutations. The restored *waaG* gene and mutant *rpoC* gene had effect on increasing the weight-average lipid length. The characterization of the cell membrane properties in the reconstructed strains provided new strategies for altering significantly the cell membrane polarization (fluidity), cell membrane lipid composition, and the cell surface hydrophobicity.

Conclusions

In this genome-level reverse engineering study, we have described the four mutations we verified in the evolved strains and the chronological order of mutations during adaptive evolution. We were able to recreate the changed phenotypic of the evolved strain by replacing mutations into the genome of the parent strain. We revealed how much each mutation and their synergistic interaction contributed on increased short-chain fatty acid tolerance, 9-fold increase in fatty acid titer, big change of EPS polysaccharides, and altering cell membrane characterizations. We noticed that the mutant *rpoC* gene plays a very important role for increasing tolerance and production, and for changing cell membrane properties. The RNA polymerase β’ subunit (*rpoC*) could impact the expression level of more than 1000 genes *E. coli.* It is very interesting to us to further investigate the *rpoC* mutation by transcriptome analysis, which will be discussed in forthcoming publications.

Acknowledgments

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Tables

Table 1. Strains used in this study

Strain	Characteristics					Reference/ Source
DH5α	Cloning host for constructing plasmids					NEB
	Mutant gene				Gene editing methods	
	waaG Insertion removed	rpoC	basR	basS		
ML115						(7)
LAR1	*	*	*			(7)
ML115+waaGInD (YC001)	*				CRISPR/Cas9 system	This Study
ML115+rpoC* (YC002)		*			Lambda Red system	This Study
ML115+basR* (YC003)			*		CRISPR/Cas9 system	This Study
ML115+basS* (YC004)				*	Lambda Red system	This Study
ML115+waaGInD+rpoC* (YC005)	*	*			CRISPR/Cas9 system	This Study
ML115+waaGInD+basR* (YC006)	*		*		CRISPR/Cas9 system	This Study
ML115+waaGInD+basS* (YC007)	*			*	CRISPR/Cas9 system	This Study
ML115+rpoC*+basR* (YC008)		*	*		CRISPR/Cas9 system	This Study
ML115+basR*+basS* (YC009)			*	*	CRISPR/Cas9 system	This Study
ML115+waaGInD+rpoC*+basR* (YC010)	*	*	*		CRISPR/Cas9 system	This Study
ML115+waaGInD+basR*+basS* (YC011)	*		*	*	CRISPR/Cas9 system	This Study
LAR1+waaGInD+rpoC+basR* (YC012)	*		*		CRISPR/Cas9 system	This Study

Table 2. Plasmids used in this study

Plasmids	Characteristics or Descriptions	Reference
pJMY-EEI82564	pTrc-EEI82564 thioesterase (TE10) from Anaerococcus tetradius, Amp^R	(7)
pKD4	FRT-Kan-FRT cassette template, Amp^R, Kan^R	(26)
pKD46	λ Red recombinase expression plasmid , Amp^R	(26)
pCP20	FLP recombinase expression, AmpR, Cm^R	(26)
pUC57-rpoC1256A	rpoC-A-FRT-Kan-FRT cassette template, Kan^R	This Study
pUC57-rpoC1256C	rpoC-C-FRT-Kan-FRE cassette template, Kan^R	This Study
pCas	*repA101*(Ts) *kan* P_{cas}-*cas9* P_{araB}-*Red lacI*^{q} P_{trc}-*sgRNA-pMB1*, Kan^R	(27)
pTarget-*pMB1*	*pMB1 aadA* sgRNA-*pMB1*	(27)
pTargetF-waaG	*pMB1 aadA* sgRNA-*waaG*-N20	This Study
pTargetF-*basS*-1	*pMB1 aadA* sgRNA-*basS*-N20-1	This Study
pTargetF-*basS*-2	*pMB1 aadA* sgRNA-*basS*-N20-2	This Study
pTargetF-*basR*-1	*pMB1 aadA* sgRNA-*basR*-N20-1	This Study
pTargetF-*basR*-2	*pMB1 aadA* sgRNA-*basR*-N20-2	This Study

Table 3. Identified mutations in evolved strains

Strain	Gene	Gene Mutation	Protein Mutation	Polypeptide/Enzyme
LAR1	*rpoC*	A1256C	H419P	RNA polymerase subunit β'
LAR1	*basR*	G82T	D28Y	DNA-binding transcriptional dual regulator BasR
LAR1	*waaG*	768 bp IS removed	3 transposase removed	lipopolysaccharide glucosyltransferase I
LAR2	*rpoC*	A1256C	H419P	RNA polymerase subunit β'
LAR2	*basS*	27 bp deletion	9 amino acids deletion	Sensory histidine kinase BasS
LAR2	*waaG*	768 bp IS removed	3 transposase removed	lipopolysaccharide glucosyltransferase I

Table 4. Contribution of mutations to increased C8 tolerance phenotype
"*" indicates that gene in the specific strain is the mutant-type gene.
[a]Log phase: recorded the hour when cell growth get into log phase, and the hour of last time point in the log phase.
[b]Inflection OD: recorded the value of OD_{550} and the hour of first time point in the stationary phase.
[c]24 h OD: recorded the value of OD_{550} at 24 hours.
ND: Not Detected.

Strain	Gene				0 mM C8				10 mM C8			
	waaG Insertion Removed	*rpoC*	*basR*	*basS*	Specific Growth Rate	Log Phase[a]	Inflection OD[b]	24 h OD[c]	Specific Growth Rate	Log Phase[a]	Inflection OD[b]	24 h OD[c]
LAR1	*	*	*		0.56±0.00	3~7 h	2.53±0.07 (8 h)	3.01±0.04	0.56±0.00	4~8 h	2.34±0.04 (9 h)	2.20±0.07
ML115					0.60±0.01	3~6 h	2.04±0.07 (7 h)	3.17±0.07	0.15±0.00	ND	ND	0.29±0.01
YC001	*				0.53±0.01	3~7 h	1.99±0.02 (8 h)	2.87±0.17	0.39±0.01	6~11 h	1.66±0.03 (12 h)	1.57±0.03
YC005	*	*			0.57±0.01	3~7 h	2.47±0.00 (8)	3.07±0.11	0.55±0.02	4~8 h	2.31±0.06 (9 h)	2.20±0.02
YC010	*	*	*		0.57±0.00	3~7 h	2.55±0.03 (8 h)	2.98±0.04	0.57±0.01	4~8 h	2.20±0.03 (9 h)	2.13±0.01
YC002		*			0.65±0.01	3~6 h	1.95±0.04 (7 h)	3.00±0.02	0.23±0.01	ND	ND	0.38±0.02
YC003			*		0.60±0.00	3~6 h	1.90±0.02 (7 h)	2.61±0.02	0.15±0.00	ND	ND	0.28±0.01
YC004				*	0.60±0.01	3~6 h	1.96±0.04 (7 h)	2.74±0.02	0.16±0.00	ND	ND	0.31±0.01
YC006	*		*		0.45±0.01	4~8 h	2.39±0.02 (9 h)	3.00±0.10	0.35±0.01	6~11 h	1.92±0.02 (12 h)	1.57±0.07
YC007	*			*	0.52±0.01	3~7 h	2.25±0.03 (8 h)	2.95±0.12	0.35±0.01	5~9 h	1.56±0.08 (10 h)	1.30±0.08
YC008		*	*		0.67±0.00	2~5 h	1.98±0.01 (6 h)	2.88±0.05	0.16±0.00	ND	ND	0.17±0.02
YC009			*	*	0.54±0.00	3~7 h	1.94±0.04 (8 h)	2.34±0.09	0.20±0.01	ND	ND	0.21±0.01
YC011	*		*	*	0.47±0.01	3~8 h	2.44±0.03 (9 h)	2.98±0.04	0.39±0.00	4~10 h	1.76±0.02 (11 h)	1.53±0.03

Figures

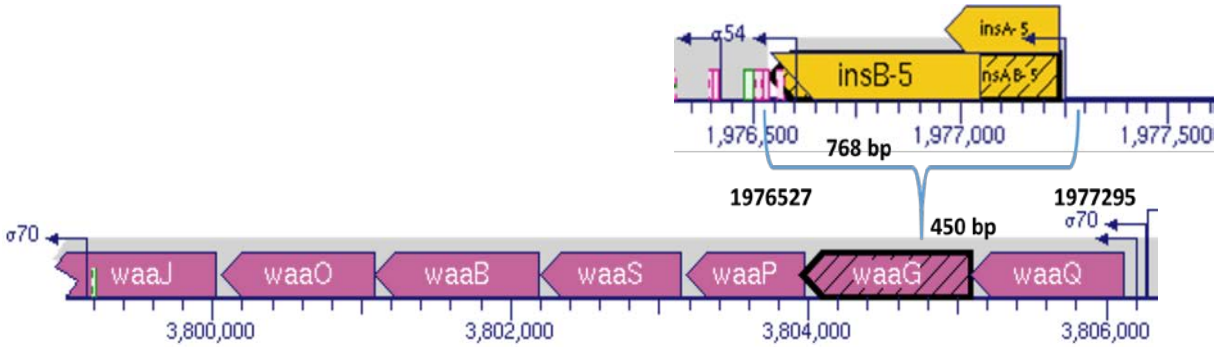
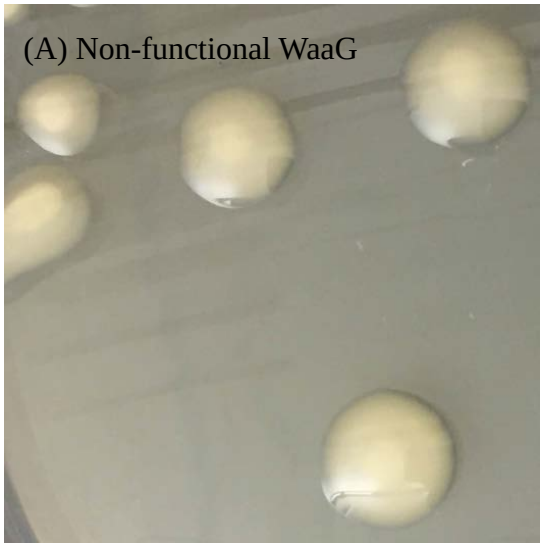
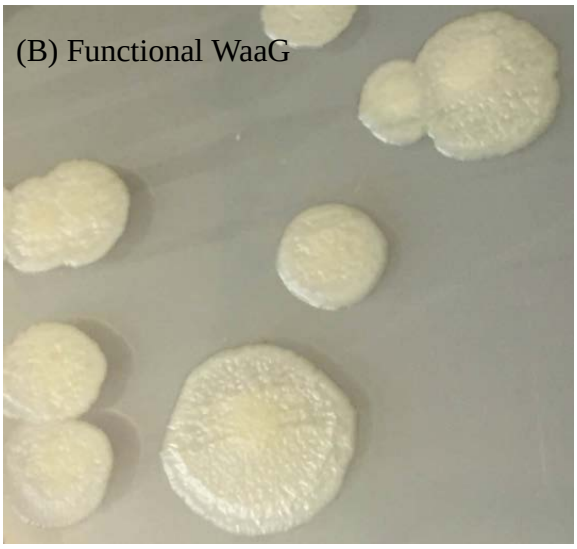


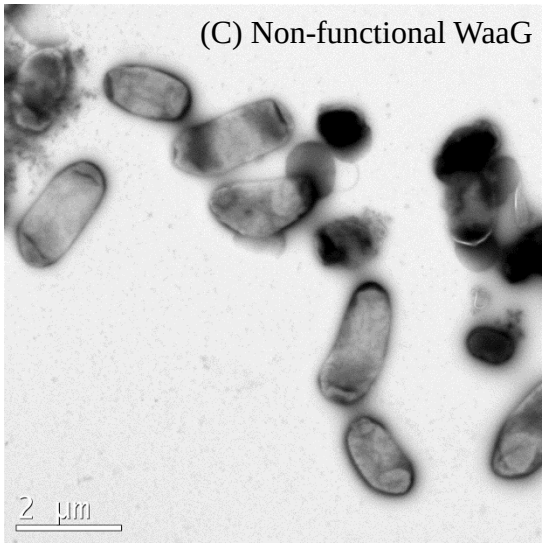
Figure 1. Parent strain ML115 has a 768 bp insertion sequence in the *waaG* gene (45).



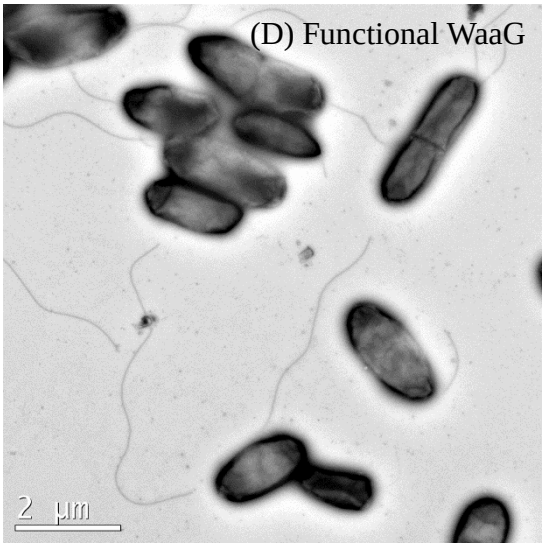
Colony morphology of ML115



Colony morphology of LAR1



TEM image of ML115



TEM image of LAR1

Figure 2. The colony morphology and TEM images of the parent strain ML115 and evolved strain LAR1.
(A) the colony morphology of ML115; (B) the colony morphology of LAR1; (C) The TEM image of ML115; (D) the TEM image of LAR1.

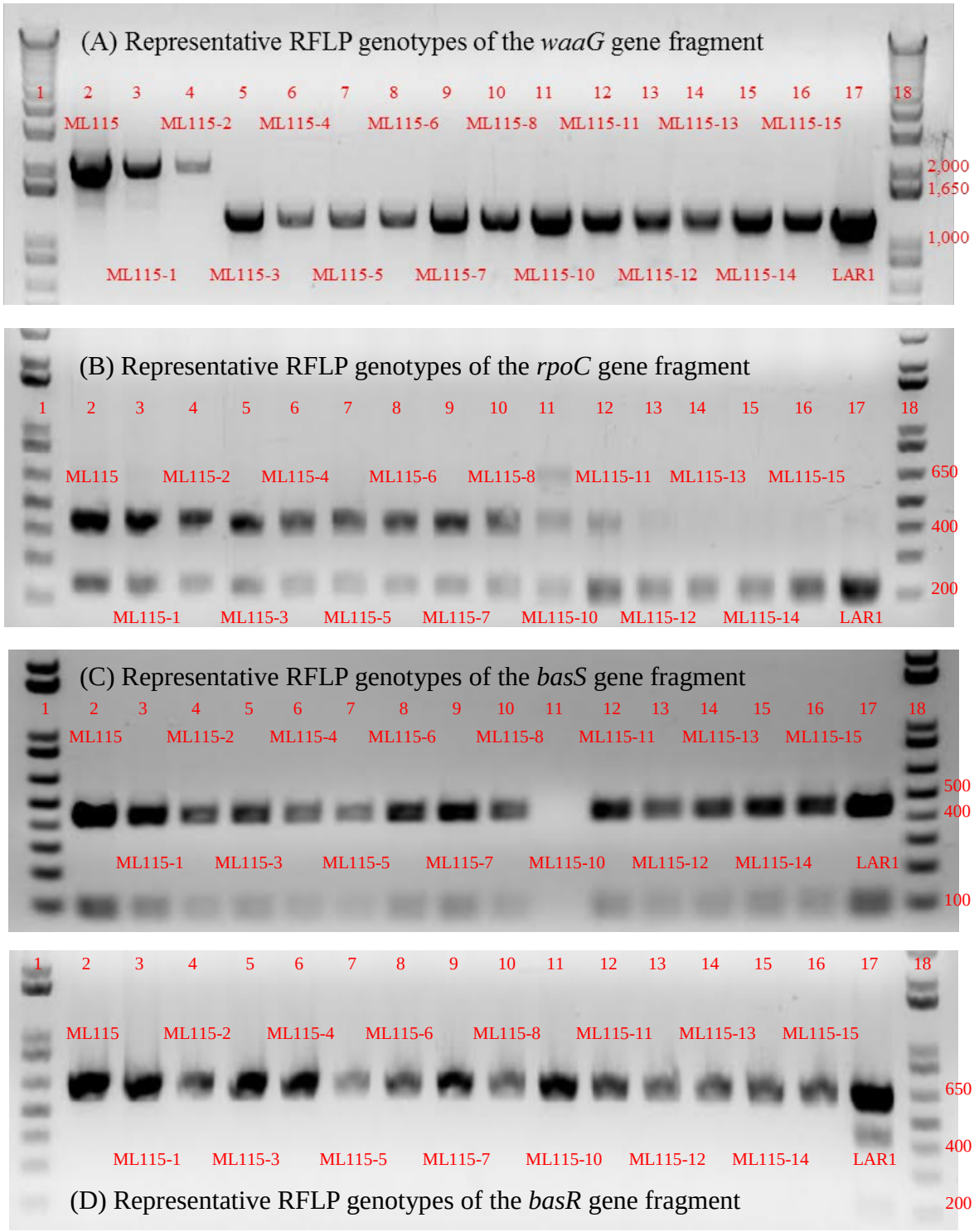

(A) Representative RFLP genotypes of the waaG gene fragment
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ML115 ML115-2 ML115-4 ML115-6 ML115-8 ML115-11 ML115-13 ML115-15
ML115-1 ML115-3 ML115-5 ML115-7 ML115-10 ML115-12 ML115-14 LAR1
2,000
1,650
1,000
(B) Representative RFLP genotypes of the rpoC gene fragment
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ML115 ML115-2 ML115-4 ML115-6 ML115-8 ML115-11 ML115-13 ML115-15
ML115-1 ML115-3 ML115-5 ML115-7 ML115-10 ML115-12 ML115-14 LAR1
650
400
200
(C) Representative RFLP genotypes of the basS gene fragment
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ML115 ML115-2 ML115-4 ML115-6 ML115-8 ML115-11 ML115-13 ML115-15
ML115-1 ML115-3 ML115-5 ML115-7 ML115-10 ML115-12 ML115-14 LAR1
500
400
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ML115 ML115-2 ML115-4 ML115-6 ML115-8 ML115-11 ML115-13 ML115-15
ML115-1 ML115-3 ML115-5 ML115-7 ML115-10 ML115-12 ML115-14 LAR1
650
400
200
(D) Representative RFLP genotypes of the basR gene fragment

Figure 3. PCR-Restriction Fragment Length Polymorphism uses to determine the order of mutations in evolved strains. Representative RFLP genotypes of the *waaG*, *rpoC*, *basS*, and *basR* genes fragment analysis using 1% agarose gel electrophoresis.
Lanes 1 and 18 refer to the 1 Kb Plus DNA Ladder (Invitrogen); lane 2 refers to the genotype of parent strain ML115; from lanes 3 to lanes 16 refers to the genotypes of cell populations which from the culture we saved before each serial transfer during adaptive evolution; lane 17 refers to the genotype of evolved strain LAR1. (A) The *waaG* mutation; (B) The *rpoC* mutation; (C) The *basS* mutation; (D) The *basR* mutation.

(A) The *rpoC* mutation Restriction enzyme: BsaJI

The sequence of the wild-type *rpoC* (427/233)

CCGGTCGTTCTGTAATCACCGTAGGTCCATACCTGCGTCTGCATCAGTGCGGTCTGCCGAAGAAAATGGCACTGGA
GCTGTTCAAACCGTTCATCTACGGCAAGCTGGAACTGCGTGGTCTTGCTACCACCATTAAAGCTGCGAAGAAAATGG
TTGAGCGCGAAGAAGCTGTCGTTTGGGATATCCTGGACGAAGTTATCCGCGAACACCCGGTACTGCTGAACCGTGC
ACCGACTCTGCACCGTCTGGGTATCCAGGCATTTGAACCGGTACTGATCGAAGGTAAAGCTATCCAGCTGCACCCGC
TGGTTTGTGCGGCATATAACGCCGACTTCGATGGTGACCAGATGGCTGTTCACGTACCGCTGACGCTGGAAGCCCA
GCTGGAAGCGCGTGCGCTGATGATGTCTACCAACAACATCCTGTCCCCGGCGAACGGCGAACCAATCATCGTTCCG
TCTCAGGACGTTGTACTGGGTCTGTACTACATGACCCGTGACTGTGTTAACGCCAAAGGCGAAGGCATGGTGCTGA
CTGGCCCGAAAGAAGCAGAACGTCTGTATCGCTCTGGTCTGGCTTCTCTGCATGCGCGCGTTAAAGTGCGTATCACC
GAGTATGAAAAAGATGCTAACGGTGAATTAGTAGCGAAAACCAGCCTGA

The sequence of the mutant *rpoC* (208/219/233)

CCGGTCGTTCTGTAATCACCGTAGGTCCATACCTGCGTCTGCATCAGTGCGGTCTGCCGAAGAAAATGGCACTGGA
GCTGTTCAAACCGTTCATCTACGGCAAGCTGGAACTGCGTGGTCTTGCTACCACCATTAAAGCTGCGAAGAAAATGG
TTGAGCGCGAAGAAGCTGTCGTTTGGGATATCCTGGACGAAGTTATCCGCGAACCCCCGGTACTGCTGAACCGTGC
ACCGACTCTGCACCGTCTGGGTATCCAGGCATTTGAACCGGTACTGATCGAAGGTAAAGCTATCCAGCTGCACCCGC
TGGTTTGTGCGGCATATAACGCCGACTTCGATGGTGACCAGATGGCTGTTCACGTACCGCTGACGCTGGAAGCCCA
GCTGGAAGCGCGTGCGCTGATGATGTCTACCAACAACATCCTGTCCCCGGCGAACGGCGAACCAATCATCGTTCCG
TCTCAGGACGTTGTACTGGGTCTGTACTACATGACCCGTGACTGTGTTAACGCCAAAGGCGAAGGCATGGTGCTGA
CTGGCCCGAAAGAAGCAGAACGTCTGTATCGCTCTGGTCTGGCTTCTCTGCATGCGCGCGTTAAAGTGCGTATCACC
GAGTATGAAAAAGATGCTAACGGTGAATTAGTAGCGAAAACCAGCCTGA

(B) The *basR* mutation Restriction enzyme: SfcI

The sequence of the wild-type *basR* (619)

CGCAAACGCAACACTATTCACAAGACAATTTATTCTCCACGCTATTGGGATTAACTGGCGTTGAGACGAAGTATTAC
CAGGCTGCGGATGATATTCTGCAAACTTGCAGGAGAGTGAGTGAatgAAAATTCTGATTGTTGAAGACGATACGCTG
TTATTGCAGGGACTGATTCTGGCGGCGCAAACCGAAGGCTACGCGTGCGATAGCGTGACAACCGCGCGGATGGCG
GAACAAAGCCTTGAGGCAGGTCATTACAGCCTGGTGGTACTGGATTTAGGGTTACCCGACGAAGATGGACTGCATT
TTCTCGCCCGTATCCGGCAGAAAAAATATACCCTGCCGGTACTGATCCTCACCGCTCGCGATACGCTGACCGACAAA
ATCGCCGGGCTGGATGTCGGTGCCGACGACTATCTGGTGAAGCCTTTTGCGCTGGAAGAGTTACATGCCCGTATCC
GCGCCCTGCTACGACGCCATAATAATCAGGGCGAAAGTGAGCTGATTGTTGGCAATCTGACGCTGAACATGGGTCG
CCGTCAGGTATGGATGGGCGGTGAAGAGTTGATTCTGACGCCCAAAGAATATGCTCTGCTGTCACGGTTAATGCTC
AAAGCAGGC

The sequence of the mutant *basR* (202/417)

CGCAAACGCAACACTATTCACAAGACAATTTATTCTCCACGCTATTGGGATTAACTGGCGTTGAGACGAAGTATTAC
CAGGCTGCGGATGATATTCTGCAAACTTGCAGGAGAGTGAGTGAatgAAAATTCTGATTGTTGAAGACGATACGCTG
TTATTGCAGGGACTGATTCTGGCGGCGCAAACCGAAGGCTACGCGTGCTATAGCGTGACAACCGCGCGGATGGCG
GAACAAAGCCTTGAGGCAGGTCATTACAGCCTGGTGGTACTGGATTTAGGGTTACCCGACGAAGATGGACTGCATT
TTCTCGCCCGTATCCGGCAGAAAAAATATACCCTGCCGGTACTGATCCTCACCGCTCGCGATACGCTGACCGACAAA
ATCGCCGGGCTGGATGTCGGTGCCGACGACTATCTGGTGAAGCCTTTTGCGCTGGAAGAGTTACATGCCCGTATCC
GCGCCCTGCTACGACGCCATAATAATCAGGGCGAAAGTGAGCTGATTGTTGGCAATCTGACGCTGAACATGGGTCG
CCGTCAGGTATGGATGGGCGGTGAAGAGTTGATTCTGACGCCCAAAGAATATGCTCTGCTGTCACGGTTAATGCTC
AAAGCAGGC

(C) The *basS* mutation Restriction enzyme: FatI

The sequence of the wild-type *basS* (84/430)

GCGAAACCTGGTAGAAAACGCCCATCGTTACAGCCCGCAAGGCAGCAACATTATGATTAAGCTGCAAGAAGATGAC
GGA|**GCGGTCATGG**CAGT**TGAAGATGAAGGA**|CCAGGTATTGATGAGAGTAAATGCGGGGAGTTGAGTAAAGCG
TTTGTACGTATGGACAGCCGTTATGGCGGGATTGGTCTGGGGTTAAGTATTGTCAGCCGCATTACACAGTTGCATCA
CGGGCAGTTTTTCCTGCAAAACCGGCAAGAGACTTCCGGCACGCGGGCCTGGGTACGGCTGAAGAAAGATCAGTA
CGTGGCAAACCAGATAtaaAGAAAGCTGCTGACCACCAGCACGCTGAACACGGTAGTTAAACTTTCATAAACACGGT
TTTTCATTGCACTCTCCTCCGTTAACCTGGAGGAGAGTATGCGCGTCAGAGATTAAGTGAACCTTAAGAGTTCAATT
GGCCTGATAAGACAGCGTCACATCAGGCCATCCGTTTCAGCTTATTCATCAATTCGCGGATGTT

The sequence of the mutant *basS* (518)

GCGAAACCTGGTAGAAAACGCCCATCGTTACAGCCCGCAAGGCAGCAACATTATGATTAAGCTGCAAGAAGATGAC
GGA|CCAGGTATTGATGAGAGTAAATGCGGGGAGTTGAGTAAAGCGTTTGTACGTATGGACAGCCGTTATGGCGG
GATTGGTCTGGGGTTAAGTATTGTCAGCCGCATTACACAGTTGCATCACGGGCAGTTTTTCCTGCAAAACCGGCAAG
AGACTTCCGGCACGCGGGCCTGGGTACGGCTGAAGAAAGATCAGTACGTGGCAAACCAGATAtaaAGAAAGCTGCT
GACCACCAGCACGCTGAACACGGTAGTTAAACTTTCATAAACACGGTTTTTCATTGCACTCTCCTCCGTTAACCTGGA
GGAGAGTATGCGCGTCAGAGATTAAGTGAACCTTAAGAGTTCAATTGGCCTGATAAGACAGCGTCACATCAGGCCA
TCCGTTTCAGCTTATTCATCAATTCGCGGATGTT

Figure 4. Design strategy for applying PCR-Restriction Fragment Length Polymorphism to determine the order of mutations in evolved strains.
(A) The *rpoC* mutation; (B) The *basS* mutation; (C) The *basR* mutation. Each mutation has a specific restriction enzyme, which can be used to determine if the gene is mutant or not. The primers for PCR are highlighted in purple; blue fonts are the sequences which can be cleaved by the specific restriction enzyme; bold fonts are the mutant positions.

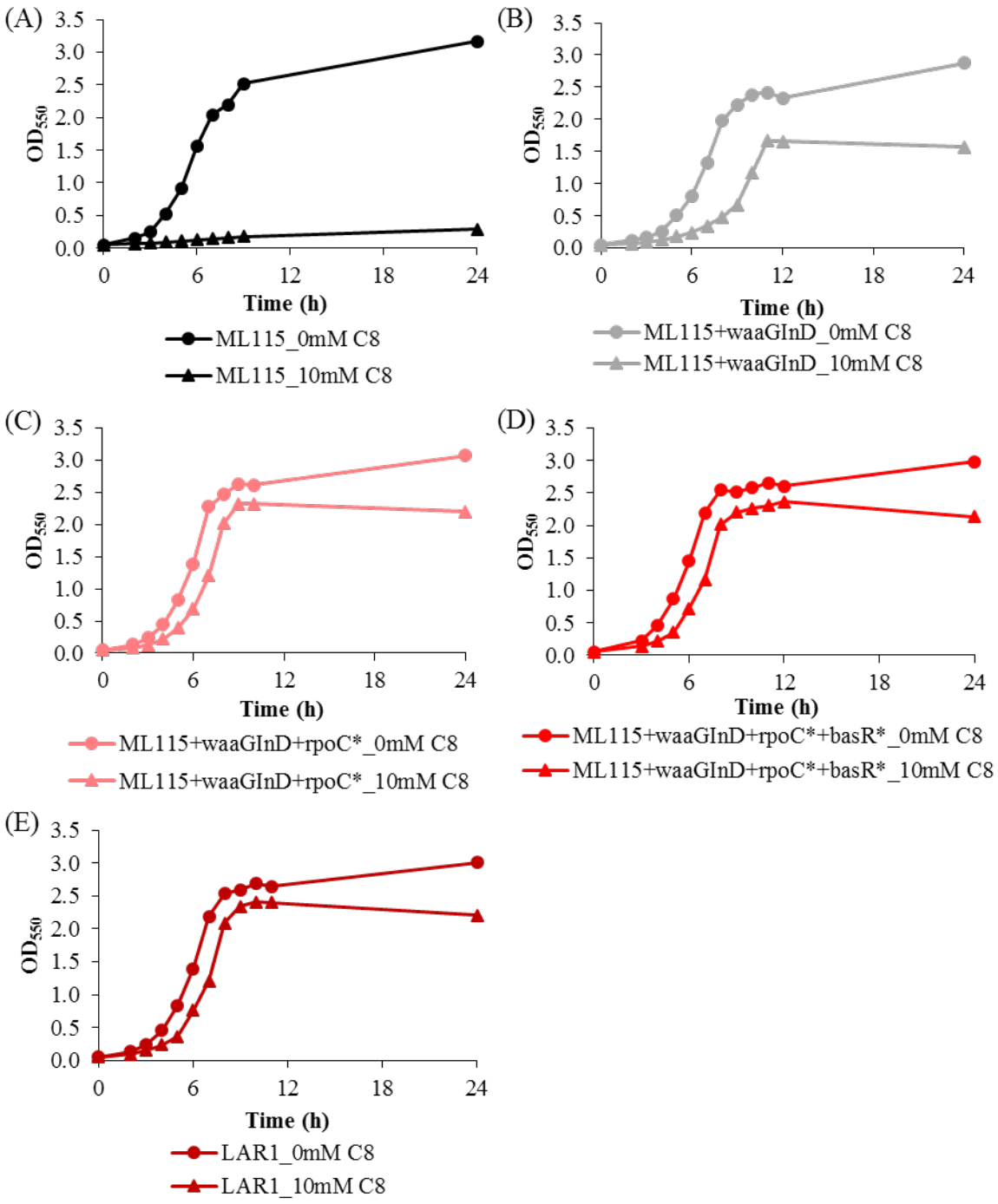


Figure 5. Exogenous 10 mM C8 tolerance test of (A) ML115; (B) ML115+waaGInD; (C) ML115+waaGInD+rpoC*; (D) ML115+waaGInD+rpoC*+basR*; E) LAR1.
Growth curve of all strains were measured in MOPS with 2.0 wt% dextrose with or without exogenously 10 mM C8 at pH 7.0, 200 rpm, and 37 ºC.

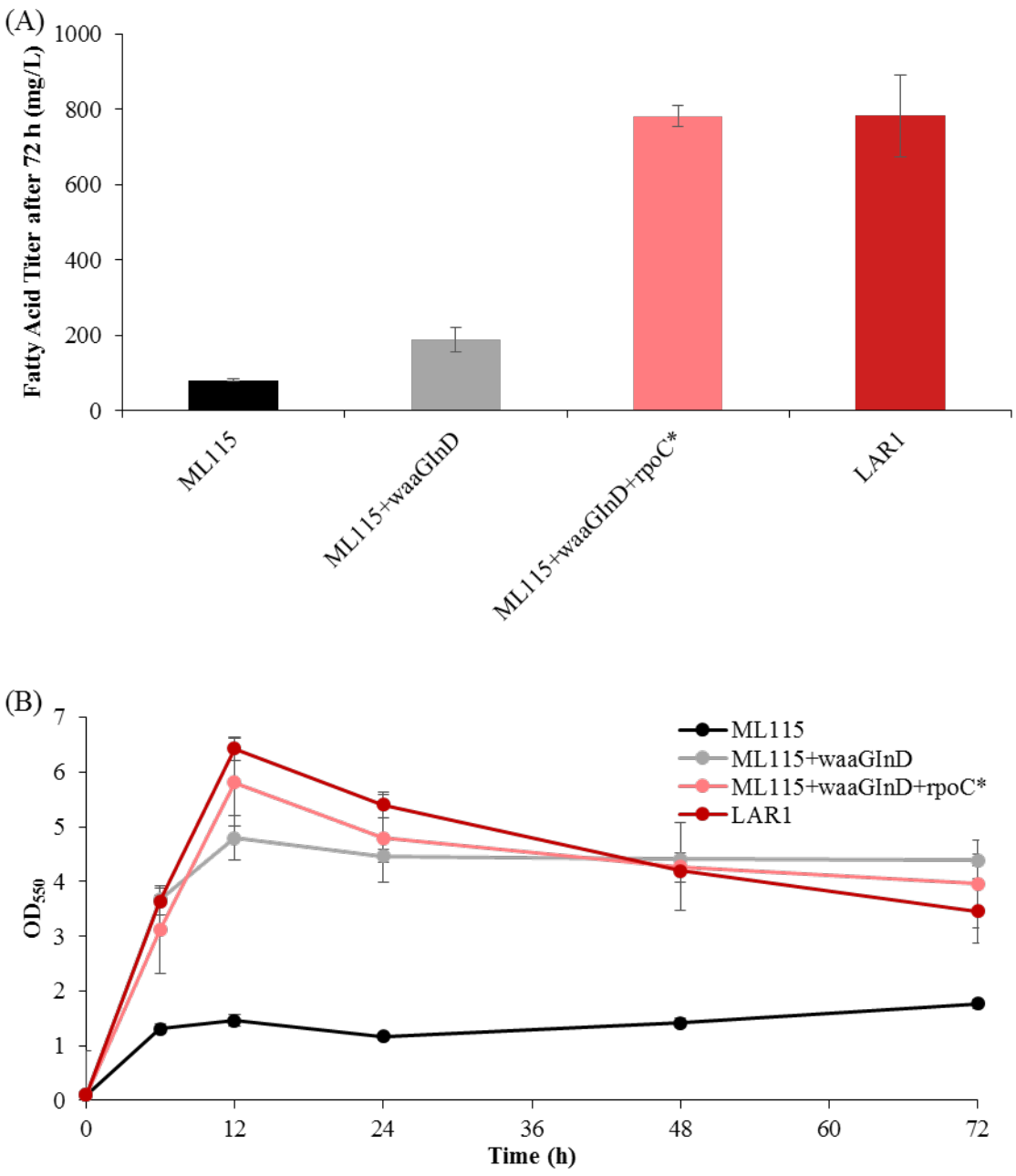


Figure 6. The restored *waaG* gene and mutant *rpoC* gene contribute to the increased fatty acid titer of the evolved strain LAR1.
Four strains contain plasmid pJMY-EEI82564 encoding the *A. tetradius* thioesterase (TE10). Strains were grown for 72 h in LB with 1.5 wt% dextrose at 30 ºC, 250 rpm with 100 mg/L ampicillin and 1.0 mM IPTG. (A) Fatty acid titer after 72 hours. (B) Strain growth during 72 hours. Values are the average of three biological replicates, with error bars indicating one standard deviation.

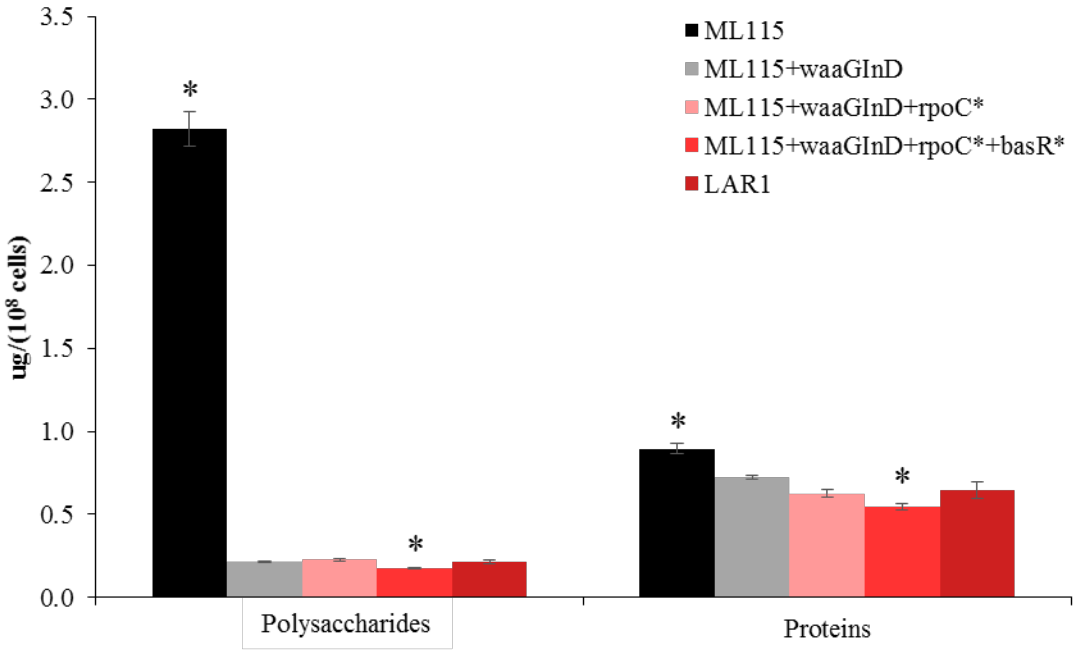


Figure 7. The extracellular polymeric substances (EPS) analysis of ML115, ML115+waaGInD, ML115+waaGInD+rpoC*, ML115+waaGInD+rpoC*+basR*, and LAR1.
The parent strain ML115 with disturbed *waaG* gene had more than 10-fold extracellular polymeric polysaccharides relative to the strains with restored *waaG* gene.* Indicates a significant difference ($p \leq 0.05$) between LAR1 with other strains.

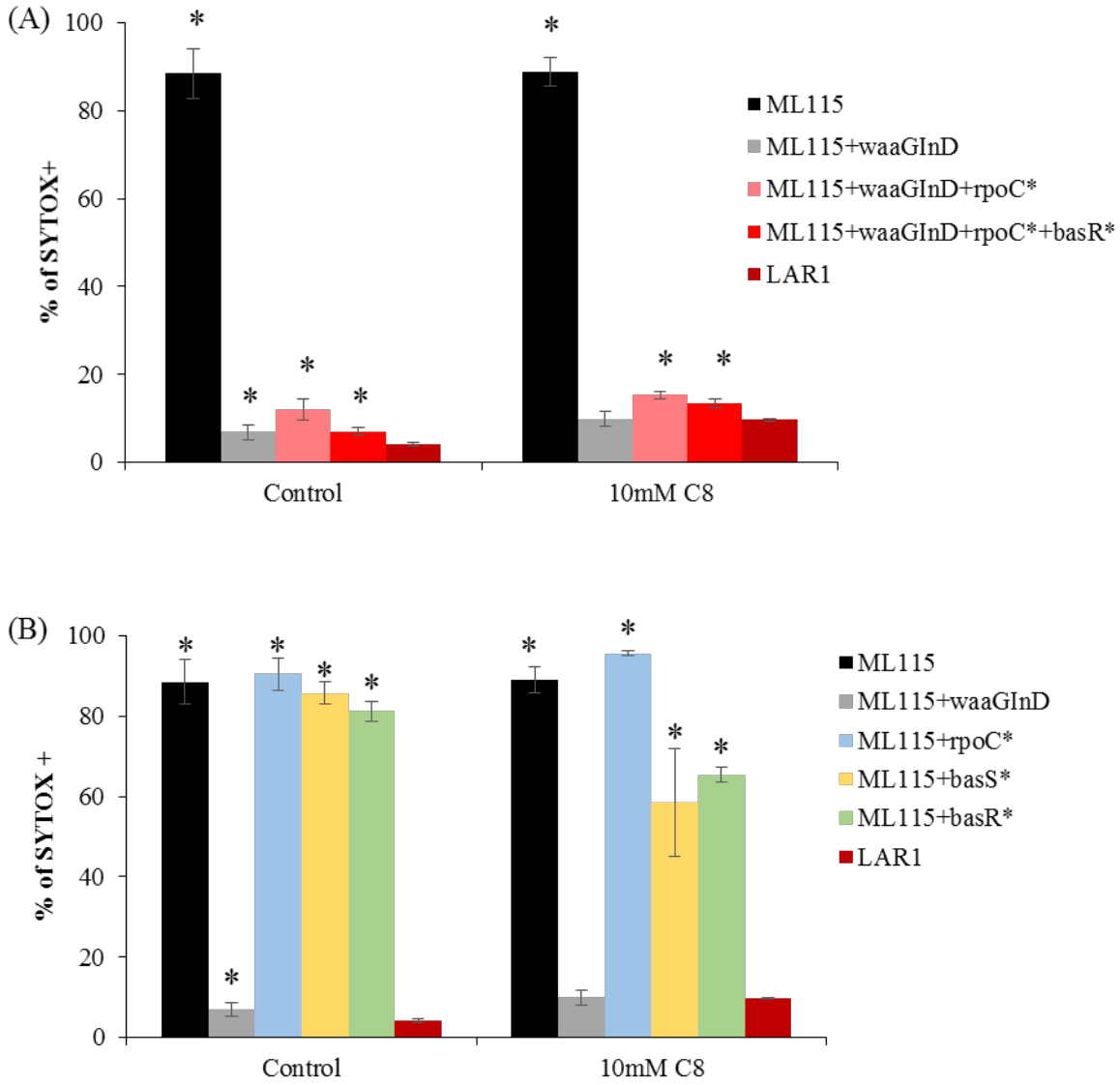


Figure 8. The cell membrane leakage of (A) ML115, ML115+waaGInD, ML115+waaGInD+rpoC*, ML115+waaGInD+rpoC*+basR*, and LAR1; (B) ML115, the constructed strains with each single mutant gene, and LAR1.
The cell membrane leakage problem was solved by repairing the non-funational WaaG; the mutant *basS** and *basR** partially contributes on solving the membrane leakage problem when treating cells with exogenous 10 mM C8 challenge. * Indicates a significant difference ($p \leq 0.05$) between LAR1 with other strains.

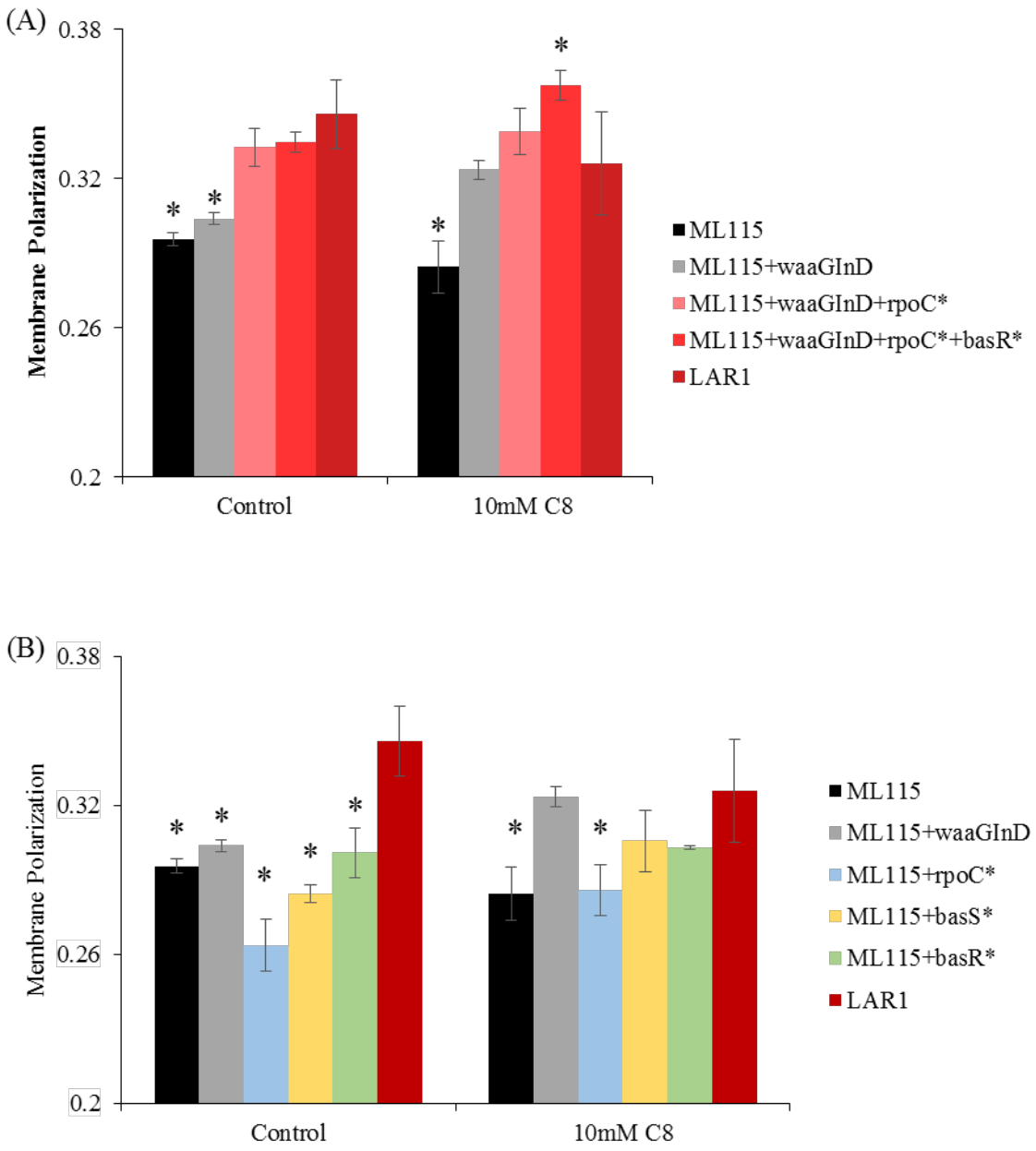


Figure 9. The cell membrane polarization of (A) ML115, ML115+waaGInD, ML115+waaGInD+rpoC*, ML115+waaGInD+rpoC*+basR*, and LAR1; (B) ML115, the constructed strains with each single mutant gene, and LAR1.
The difference of cell membrane polarization between ML115 and LAR1 was contributed by the synergistic interaction among the 3 mutations. * Indicates a significant difference ($p \leq 0.05$) between LAR1 with other strains.

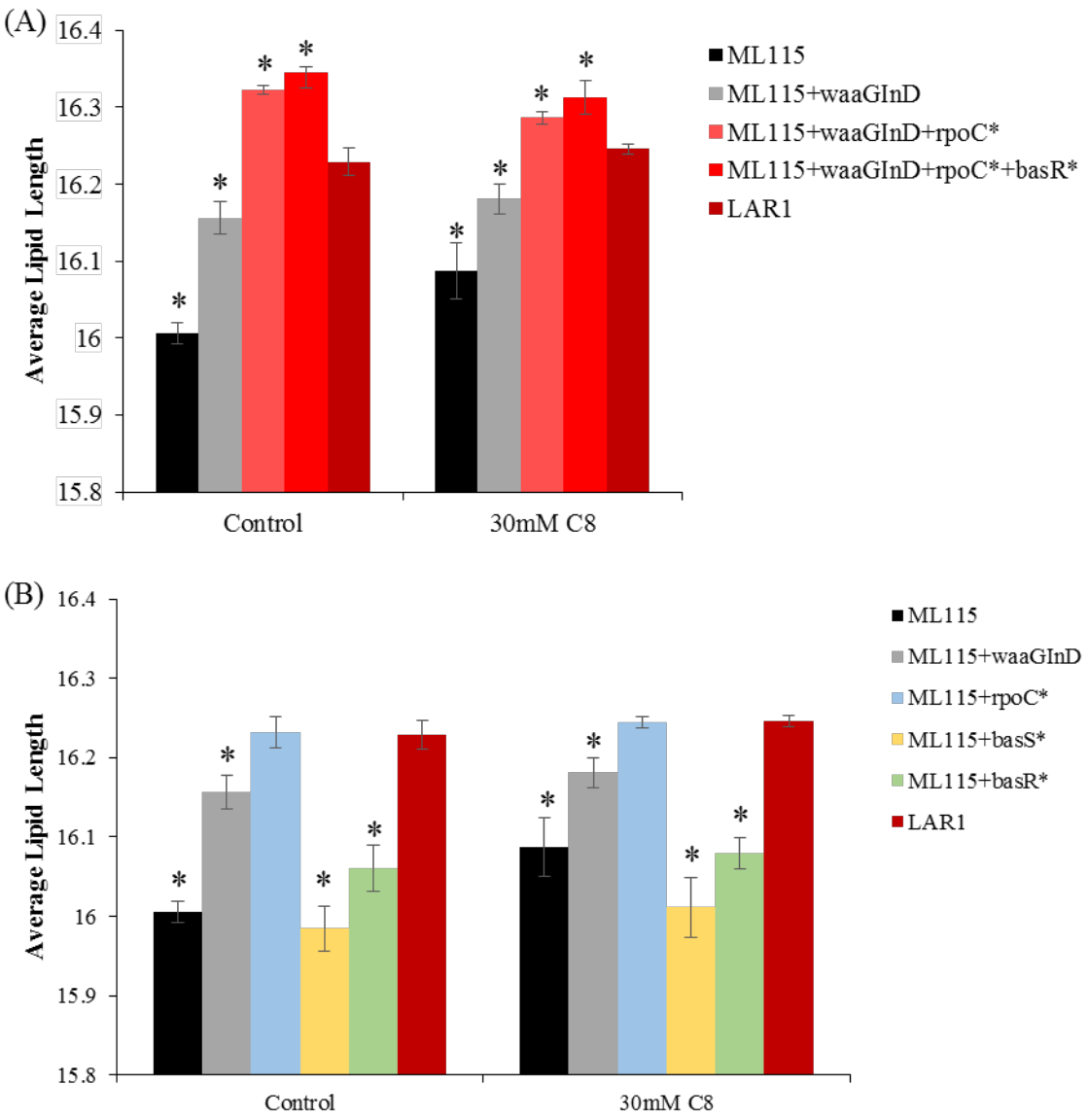


Figure 10. The weight-average lipid length of (A) ML115, ML115+waaGInD, ML115+waaGInD+rpoC*, ML115+waaGInD+rpoC*+basR*, and LAR1; (B) ML115, the constructed strains with each single mutant gene, and LAR1.
The restored *waaG* gene and mutant *rpoC* gene both contributes to increased weight-average lipid length of evolved strain LAR1. * Indicates a significant difference ($p \leq 0.05$) between LAR1 with other strains

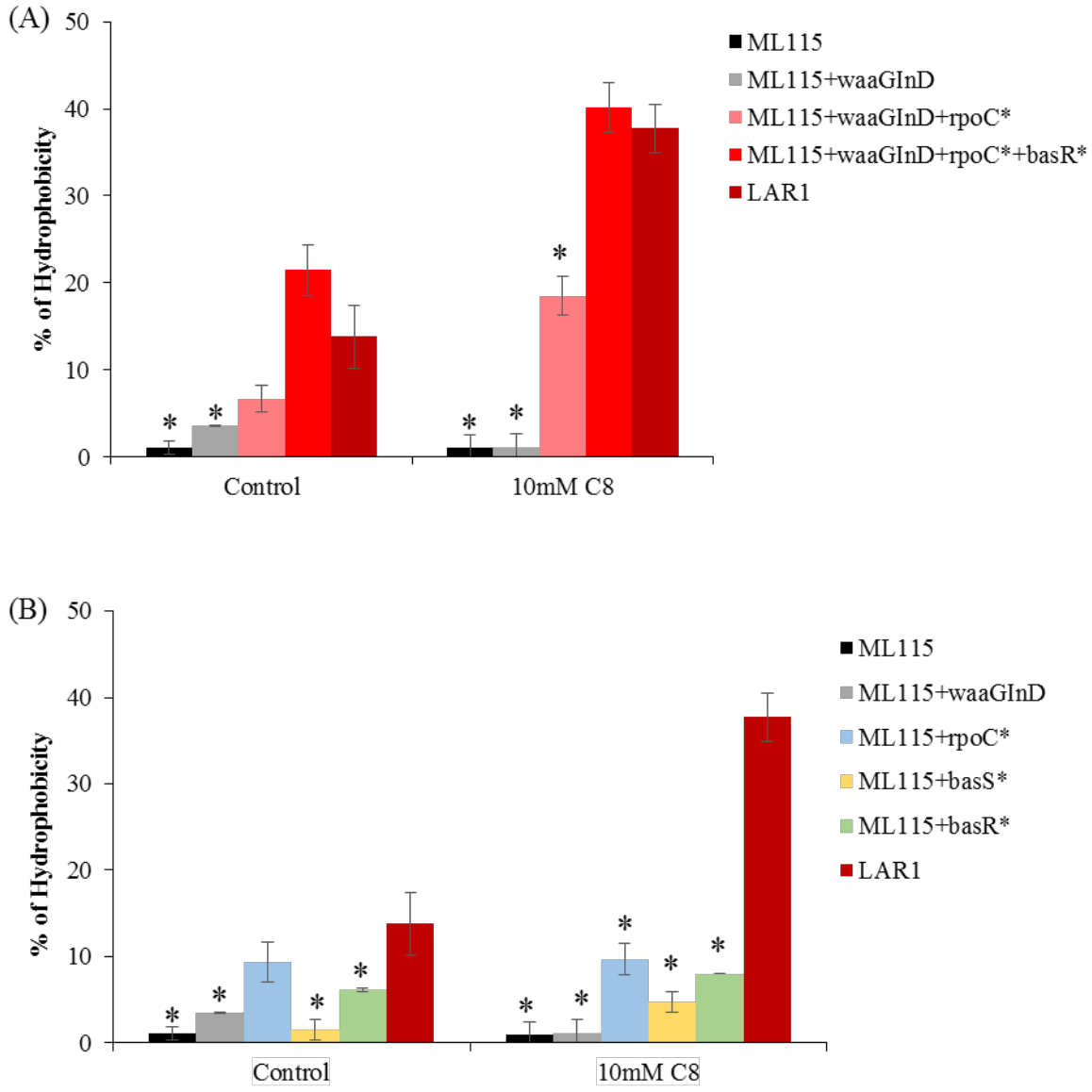


Figure 11. The cell surface hydrophobicity of (A) ML115, ML115+waaGInD, ML115+waaGInD+rpoC*, ML115+waaGInD+rpoC*+basR*, and LAR1; (B) ML115, the constructed strains with each single mutant gene, and LAR1.
The difference of cell surface hydrophobicity between ML115 and LAR1 was contributed by the synergistic interaction of the 3 mutations.
* Indicates a significant difference ($p \leq 0.05$) between LAR1 with other strains.

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CHAPTER 5. TRANSCRIPTOMIC ANALYSIS OF *ESCHERICHIA COLI* EVOLVED FOR SHORT-CHAIN FATTY ACID

Draft of a research paper to be submitted to *Metabolic Engineering*

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Introduction

Engineering microbial biocatalyst for producing biorenewable fuels and chemicals is an increasingly important area of research. Progress in biorenewable fuel and chemical research can contribute to a decreased reliance on petroleum derived compounds. Fatty acids are a valuable biorenewable chemical in industrial application as multifunctional precursors, which be converted to alkanes (1, 2), α-olefins (3, 4), and fatty acid methyl or ethyl esters (5). Short-chain fatty acids (SCFAs) are also directly used as food-preservatives (6, 7) and dietary supplements (8). As a food-preservative, SCFAs are toxic to microbes, which could inhibit cell growth and the fermentation performance (9–14). Many efforts have been made to address *E. coli* inhibition by SCFAs (9–12, 15). Our group focused on improving the SCFAs tolerance and production in *E. coli* by adaptive laboratory evolution (10) and understanding the mechanisms of inhibition and increased tolerance by reverse engineering.

After the adaptive laboratory evolution, we obtained an evolved strain LAR1, which had a significant increase in SCFAs (C6-C10) tolerance and could produce at least 5-fold fatty acids relative to the parent strain ML115 (10). In order to understand the mechanisms of the beneficial phenotypes of the evolved strain, we applied the genome-level reverse engineering for further

investigation. We identified three mutations in LAR1 strain and studied the contributions of each mutations or their synergistic interaction. We found that the RpoC (H419) mutation contributed to the increased C8 tolerance and the increased fatty acid titer. The BasR (D28Y) mutation was able to partially mitigate the membrane leakage problem of cells in 10 mM C8 and the BasR (D28Y) mutation also increased the cell surface hydrophobicity. However, the two mutations encode transcriptional regulators, which cloud results in altering expression level of many genes in LAR1 (16–19).Transcriptome analysis is a powerful tool that has been used in the investigation of evolved strains, especially the evolved strains with mutations related to regulators (20–23).Thus, we decided to utilize transcriptome analysis for further investigation of the evolved strain LAR1. In this work, we aimed to reveal how the *rpoC* and *basR* mutations lead to phenotypic changes in short-chain fatty acid-tolerance.

Materials and Methods

Strains, plasmids and bacterial cultivation

All bacterial strains and plasmids used in this study are listed in Table 1 and Table2. All *E. coli* strains were grown overnight in 10 mL of MOPS minimal media with 2% glucose in 250 mL flasks, at 37 ºC with 250 rpm. The overnight culture was diluted to OD_{550} of 0.05 for the octanoic acid (C8) tolerance test, or diluted to OD_{550} of 0.1 for testing membrane leakage, membrane fluidity, cell hydrophobicity, and cell membrane composition. *E. coli* transformants were grown in media at 37 ºC or 30 ºC, with chloramphenicol (35 mg/L), ampicillin (100 mg/L), kanamycin (50 mg/L), or spectinomycin (50 mg/L) as needed.

Genomic manipulations

The scarless CRISPR-Cas9 approach was applied to knock out the genes in LAR1 and ML115+waaGInD strains (24).

Octanoic acid tolerance test

Octanoic acid tolerance was determined follow the same procedures in Chapter 4.

Fermentation for fatty acid production

The fatty acid production strains harboring the pJMY-EEI82564 plasmid (10) by electroporation were grown on LB plates with ampicillin (100 mg/L) and incubated at 30 ºC overnight. Individual colonies were inoculated into 10 mL LB media with ampicillin (100 mg/L) in 250 mL flasks at 30 ºC and 250 rpm for overnight. Seed cultures were then inoculated into 500 mL flasks containing 100 mL of LB media with 1.5% dextrose, ampicillin (100 mg/L), and isopropyl-β-D-thiogalactopyranoside (IPTG) (1.0 mM) at an initial OD_{550} of 0.1. The flasks were incubated in a rotary shaker at 200 rpm and 30 ºC for 72 hours.

Two mL of culture samples at 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h were saved in -20 ºC refrigerator for testing fatty acid titer and glucose consumption. OD_{550} and pH of media was tested at each time point. Simultaneously, saved cell pellets (OD_{550}=1.5) were first frozen in liquid nitrogen and then stored in -80˚C for total RNA isolation.

Determination of fatty acid titers

Fatty acid extraction and derivatization follow the same procedures in Chapter 4, as well as the GC-MS analysis (25).

Determination of glucose consumption

One mL saved culture samples were first centrifuged at 1450×g for 3 min, and the supernatant were filtered (0.45 μm pore size) followed by HPLC analysis. The HPLC system equipped with Aminex HPX-87H column and RID detector (Waters, Milford, MA). The program was as follows: 5 mM sulfuric acid was used as mobile phase; flow rate was 0.3 mL/min; sample size was 10 μL; column temperature at 30 ºC; and running time was 30

min/sample. RID peak area were compared to a glucose standard curve to determine the concentration of glucose.

RNA isolation

Total RNA was isolated from saved cell pellets sampled at 6 h, 12 h and 24 h using RNeasy mini kit (Qiagen, Valencia, CA). Then genomic DNA contamination was removed by Turbo DNA-free kit (Life Technologies, Carlsbad, CA) followed by the verification of total RNA using Agilent 2100 Bioanalyzer, and RNA 600 Nano total RNA kit (Agilent, Santa Clara, CA). Ribosomal RNA (rRNA) was removed by Ribo-Zero (Bacteria) Magnetic kit (Illumina, San Diego, CA), and then massage RNA (mRNA) was purified and concentrated by the Rneasy MinElute Cleanup kit (Qiagen, Valencia, CA). Again, Agilent 2100 Bioanalyzer and RNA 6000 Pico mRNA kit (Agilent, Santa Clara, CA) were used to verify the mRNA sample, which should not contain rRNA. All procedures were followed the manufacturers' user guide.

Analysis of RNA-seq data

Single-end, directional RNA-Seq was performed by the Iowa State University DNA facility using the Illumina HiSeq 3000 platform with reads 100 base pairs (bp) in length. Reference-based assembly was performed using Rockhopper2 (version 2.0.3), which is designed specifically for bacterial systems (26). We created a reference transcriptome was corresponding to pJMY-EEI82564 that contained sequences of genes on the plasmid. Both *de novo* assembly and alignment to *E. coli* K-12 MG1655 (version U00096.3) (27, 28) reference transcriptome and pJMY-EEI82564 transcriptome were performed, which represented a combined 4,321 transcripts. Assembled RNA transcripts were used to further validate previously predicted genomic mutations.

Raw read counts from the Rockhopper2 assembly were analyzed with DESeq2 version 1.20.0 (29), a statistical package for differential expression analysis in R version 3.5.0.1. DESeq2 was used to normalize raw count data and to test for differential expression between conditions and calculate pairwise $\log_2$ fold change (LFC). Differential expression analysis was performed in a pair-wise manner for strain contrasts at each time point and with each plasmid treatment. Differentially expressed genes were those with False Discovery Rate (FDR) adjusted *p*-value < 0.05.

Annotations from EcoCyc (30) and RegulonDB (31) were used to identify genes with promoters associated with sigma factor 70 or belonging to the BasS-BasR regulon. Gene Ontology (GO) enrichment was performed to identify trends in gene function and localization annotations (Gene Ontology Consortium validation date 12/21/2015). Gene lists were analyzed for overrepresented GO terms in the biological process, cellular component, and molecular function ontologies using BiNGO and a significance level of FDR corrected *p*-value < 0.05.

E. coli Variant Analysis (EVA) software was used to generate a gene regulatory and metabolic network that reflected mutated features and downstream biological elements. Onto the network, we applied fold change data for strain contrasts to visualize altered transcript abundance for genes in the BasR regulon. We additionally modified EVA for use with candidate gene lists. Given a list of genes as input, we generated a gene regulatory and metabolic network to find potential interactions among perturbed genes.

Membrane characterization

Membrane permeability (32), membrane fluidity (9, 33), cell surface hydrophobicity (34, 35) and membrane fatty acid lipid composition (36) were assessed by following the same producers in Chapter 4.

Results and Discussion

Phenotypes of strains during fatty acid fermentation

We first compared the cell growth during short-chain fatty acid production of parent strain ML115 and evolved strain LAR1 with pJMY-EEI82564 or pJMY-Empty (Figure 1A). LAR1 with pJMY-EEI82564 had the highest OD_{550} after 24 hours, which was 3.2, then decreased to 2.9 after 72 hours. ML115 with pJMY-EEI82564 reached the highest OD_{550} after 12 hours, which was 1.4, then decreased to 1.3 after 72 hours. LAR1 and ML115 with pJMY-Empty reached stationary phase after 12 hours with an OD_{550} of 1.8 and 2.1, then slightly increased to a final OD_{550} of 2.2 and 2.5. The LAR1 with pJMY-EEI82546 had the fastest growth and highest OD_{550} relative to other strains, even comparing with the LAR1with pJMY-Empty, which indicated the LAR1 grew even better when the cells producing toxic short-chain fatty acid.

LAR1 strain with pJMY-EEI82564 could achieve the highest fatty acid titer 420 mg/L, which was 3-fold higher than that of ML115 with pJMY+EEI82564 (Figure 1B.) The fatty acid titer of LAR1 and ML115 with pJMY-Empty was about 35 mg/L. The strains with pJMY-EEI82564 mostly produced the free fatty acids C4, C6, C8:0, C8:1, C10:0, C10:1, C12:1, C12:0, C14:1, C14:0, C16:1, C16:0, C18:1, and C18:0, with the C8 and C16 as the primary components (Figure 1E). The strains with pJMY-Empty mostly produced free fatty acids C14:0, C16:1, C16:0, C18:1, and C18:0, with the C16:0 and C18:0 as the primary components (Figure 1E).

LAR1 and ML115 strains with pJMY-EEI82564 consumed glucose mainly between 6 to 24 hours, and the glucose consumption rate was 0.35 g/L/h and 0.14 g/L/h, respectively (Figure 1C). Contrastingly, LAR1 and ML115 strains with pJMY-Empty consumed the most glucose during the lag and log phases (0 - 24 hours), the glucose consumption rate is 0.107 g/L/h and

0.165 g/L/h, respectively. After 24 hours, the glucose consumption was stopped for all the four strains.

We also tested the pH of the fermentation media at different time points for all the strains (Figure 6D). Surprisingly, LAR1 with pJMY-EEI82564 was able to maintain the pH above 5.4, which had the highest short-chain fatty acid titer. The pH of the fermentation media of LAR1 with pJMY-Empty, LAR1 and ML115 with pJMY-EEI82564 were below 4.85 after 72 hours.

For the transcriptomic analysis, samples from the 6 hours, 12 hours, and 24 hours were selected. The three time points were selected to include one sample from each of the three main phases of growth: the lag phase, the log phase, and the stationary phase. After 24 hours, the OD_{550} value, the fatty acid titer, and the glucose concentration of the four strains were stable.

Identifying the potential impacts of *rpoC* mutation

Differentially expressed genes were identified for relation to the *rpoC* mutation by examining if they are known to be associated with sigma factor 70. Firstly, we identified genes that have a statistically significant and either consistently positive or negative fold change across at three time points between LAR1+pJMY-Empty and ML115+pJMY-Empty, and also between LAR1+pJMY-EEI82564 and ML115+pJMY-EEI82564. Fifty-eight genes had lower transcript abundance and forty-three genes had higher transcript abundance in LAR1 versus ML115. These sets of genes were filtered based on if they had a promoter that is associated with sigma factor 70, resulting in thirteen downregulated and sixteen upregulated genes (Table 3).

We decided to investigate the genes with either minimal fold changer (MFC) smaller than 0.5, or MFC larger than 1.9 (Table 3), by knocking out individual genes or the whole operons in the genome of ML115+waaGInD strain (Table 1). Surprisingly, the *waaG* gene had fold changer 0.44, which indicates the expression level of *waaG* gene is higher in the parent strain ML115

relative to the evolved strain LAR1. However, from the previous study, we identified an insertion sequence in the *waaG* gene in the genome of ML115. Further investigation of the *waaG* gene has been done and reported in Chapter 4. Thus, the *waaG* gene was excluded from the gene list for further investigation.

We considered that genes with sigma 70 promoters could also be influenced by other mutations in LAR1. We analyzed the gene regulatory network generated by EVA for the *basR* mutations and included the next three levels of gene regulation, which included 130 genes. When cross-referencing the list of genes downstream of these mutations with the differentially expressed genes mentioned above, we identified *osmY* and *fliR* genes as being indirectly regulated by the BasR transcription factor. In the case of *osmY*, BasR is a transcriptional activator for *csgD* (18). CsgD represses transcription of *fliZ* (37), and FliZ represses transcription of *csgD* and *osmY*. For *fliR*(38, 39), BasR activates transcription of *qseB* (40), QseB is believed to activate transcription of *flhC* and *flhD* (41), and FlhDC is a transcriptional activator for *fliR* (42). In both cases, it is possible that the differences in transcript abundance between strains are indirectly affected by the *basR* mutation.

GO enrichment highlighted the relationship between *fdnH* and *fdnI* as components of the formate dehydrogenase complex but did not give insight into larger trends among the genes. Because these genes are in the same operon and co-transcribed, it is not surprising that they appeared in the same list. Other operons that appeared in our analysis include *yfiRNB, fadAB,* and *hofBC*. The *hofBC* genes have sequence similarity to protein secretion and fimbrial assembly genes (43) and *yfiRNB* genes is involved in exopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. Thus, we decided to knock out the *fdnGHI, yfiRNB, fadAB, and hofBC* operons in

the genome of ML115+waaGInD strain, instead of knocking out each gene for further investigation (Table 1).

Identifying the potential impacts of *basR* mutation

Differentially expressed genes were compared with genes in the BasS-R regulon and up to three downstream levels of transcriptional regulation. In order to investigate genes in the BasR regulon, we used EVA to construct a gene regulatory and metabolic network that represented genes downstream of the transcription factor and included additional transcriptional regulation activities. Genes with consistent and statistically significant fold changes at three time points between LAR1+pJMY-EEI82564 and ML115+pJMY-EEI82564 were listed in Table 3. We decided to investigate the genes with either MFC smaller than 0.5, or MFC larger than 2.0 (Table 4), by knocking out individual genes or the whole operons in the genome of LAR1 strain (Table 1). From previous a report, overexpression of *arnB* gene in strain BW25113 significantly increased *n*-butanol tolerance (19). In our study, the analysis results showed some of the genes in *arn* operon had significant fold changes ($p \leq 0.05$) at 12 hours and /or 24 hours. We decided to knock out the *arnABCDTEF* operon in the genome of LAR1 strain for further investigation (Table 1).

Impact of Δ*bssS* on C8 tolerance

The reconstructed strain ML115+waaGInD+Δ*bssS* had higher specific growth rate in MOPS with 2 wt% glucose without C8 challenging relative to ML115+waaGInD and ML115+waaGInD+rpoC* (Figure 2A). The ML115+waaGInD+Δ*bssS* and ML115+waaGInD+rpoC* strains had similar OD_{550} at 24 hour. The specific growth rate of ML115+waaGInD+Δ*bssS* in MOPS with 2 wt% glucose with 10mM C8 is 0.44 h^{-1}, which is higher than ML115+waaGInD, but still lower than ML115+waaGInD+rpoC* (Figure 2B). The

OD_{550} of ML115+waaGInD+Δ*bssS* and ML115+waaGInD at 24 hours was approximately 1.5, which was significantly lower than the OD_{550} of ML115+waaGInD+rpoC* at the same time. When challenged with 10 mM C8, the *bssS* knockout mutant only had increased specific growth rate but did not have higher OD_{550} compared to the ML115+waaGInD strain. It was still strong evidence that knocking out the *bssS* gene in ML115+waaGInD is able to increase the C8 tolerance which could be caused by the *rpoC* mutation.

Conclusion and Further Plan

In this present work, in order to have a better understanding of the RpoC (H419) and BasR (D28Y) mutations, we further investigated the evolved strain LAR1 by transcriptome analysis during fatty acid production. Twenty-nine genes were identified that could be influenced by *rpoC* mutation, which had consistent and statistically significant fold changes between LAR1+pJMY-EEI82564 and ML115+pJMY-EEI82564, and between LAR1+pJMY-Empty and ML115+pJMY-Empty, at 6 hours, 12 hours, and 24 hours. We decided to start with studying the genes with MFC smaller than 0.50 or MFC larger than 1.90, by knocking out the genes or the operons in the genome of ML115+waaGInD strain. Nine genes were targeted that could be impacted by the *basR* mutation, which had consistent and statistically significant fold changes between LAR1+pJMY-EEI82564 and ML115+pJMY-EEI82564, at 6 hours, 12 hours, and 24 hours. The genes with MFC smaller than 0.50 or MFC larger than 2.0 were selected for further investigation. We decided to knock out the genes or the operons in the genome of the LAR1 strain.

Firstly, we will test C8 tolerance of all reconstructed strains to identify the genes impact on C8 tolerance. Then we will identify the genes which not only impact C8 tolerance but also influence on the fatty acid production, by testing the fatty acid production of the screen out

strains from tolerance study. Finally, we will further study the genes which contribute to both increased C8 tolerance and improved fatty acid production to understand the mechanisms of *rpoC* mutation and *basR* mutation.

With this study, we can identify the genes which could increase short-chain fatty acid titer and improve fatty acid production by altering the expression level. Thus, we are able to understand the mechanisms of the beneficial phenotypes of evolved strain LAR1. There are a few genes of unknown function in the interesting gene list. We may able to characterize the protein function of these genes.

Tables

Table 1. Strains used in this study

Strain	Descriptions	Reference/ Source
DH5α	Cloning host for constructing plasmids	NEB
ML115	The parent strain for C8 adaptive evolution	(10)
LAR1	ML115 evolved for C8 tolerance	(10)
ML115+waaGInD (YC001)	ML115 with wild-type *waaG*	This Study
ML115+waaGInD+Δ*bssS*	ML115 with wild-type *waaG*Δ*bssS*	This Study
ML115+waaGInD+Δ*yjbE*	ML115 with wild-type *waaG*Δ*yjbE*	This Study
ML115+waaGInD+Δ*dsrA*	ML115 with wild-type *waaG*Δ*dsrA*	This Study
ML115+waaGInD+Δ*ssrA*	ML115 with wild-type *waaG*Δ*ssrA*	This Study
ML115+waaGInD+Δ*ugpQ*	ML115 with wild-type *waaG*Δ*ugpQ*	This Study
ML115+waaGInD+Δ*osmY*	ML115 with wild-type *waaG*Δ*osmY*	This Study
ML115+waaGInD+Δ*mtn*Δ*btuF*	ML115 with wild-type *waaG*Δ*mtn*Δ*btuF*	This Study
ML115+waaGInD+Δ*fdnG*Δ*fdnH*Δ*fdnI*	ML115 with wild-type *waaG*Δ*fdnG*Δ*fdnH*Δ*fdnI*	This Study
ML115+waaGInD+Δ*pka*	ML115 with wild-type *waaG*Δ*pka*	This Study
ML115+waaGInD+Δ*yfiR*Δ*yfiN*Δ*yfiB*	ML115 with wild-type *waaG*Δ*yfiR*Δ*yfiN*Δ*yfiB*	This Study
ML115+waaGInD+Δ*fadB*Δ*fadA*	ML115 with wild-type *waaG*Δ*fadB*Δ*fadA*	This Study
ML115+waaGInD+Δ*hofC*Δ*hofB*	ML115 with wild-type *waaG*Δ*hofC*Δ*hofB*	This Study
LAR1+Δ*yjbJ*	LAR1Δ*yjbJ*	This Study
LAR1+Δ*fliC*	LAR1Δ*fliC*	This Study
LAR1+Δ*hdeA*Δ*hdeB*	LAR1Δ*hdeA*Δ*hdeB*	This Study
LAR1+Δ*mglB*Δ*mglA*Δ*mglC*	LAR1Δ*mglB*Δ*mglA*Δ*mglC*	This Study
LAR1+Δ*csgA*Δ*csgB*Δ*csgC*Δ*csgD*Δ*csgE*Δ*csgF*Δ*csgG*	LAR1Δ*csgA*Δ*csgB*Δ*csgC*Δ*csgD*Δ*csgE*Δ*csgF*Δ*csgG*	This Study
LAR1+Δ*arnA*Δ*arnB*Δ*arnC*Δ*arnD*Δ*arnT*Δ*arnE*Δ*arnF*	LAR1Δ*arnA*Δ*arnB*Δ*arnC*Δ*arnD*Δ*arnT*Δ*arnE*Δ*arnF*	This Study

Table 2. Plasmids used in this study

Plasmids	Characteristics or Descriptions	Reference
pJMY-EEI82564	pTrc-EEI82564 thioesterase (TE10) from Anaerococcus tetradius, Amp^R	(10)
pCas	*repA101*(Ts) *kan* P_{cas}-*cas9* P_{araB}-*Red lacI*^{q} P_{trc}-*sgRNA-pMB1*, Kan^R	(24)
pTarget-*pMB1*	*pMB1 aadA* sgRNA-*pMB1*	(24)
pTargetF-*bssS*	*pMB1 aadA* sgRNA-*bssS*-N20	This Study
pTargetF-*yjbE*	*pMB1 aadA* sgRNA-*yjbE*-N20	This Study
pTargetF-*dsrA*	*pMB1 aadA* sgRNA-*dsrA*-N20	This Study
pTargetF-*ssrA*	*pMB1 aadA* sgRNA-*ssrA*-N20	This Study
pTargetF-*ugpQ*	*pMB1 aadA* sgRNA-*ugpQ*-N20	This Study
pTargetF-*osmY*	*pMB1 aadA* sgRNA-*osmY*-N20	This Study
pTargetF-*btuF*	*pMB1 aadA* sgRNA-*btuF*-N20	This Study
pTargetF-*fdnG*	*pMB1 aadA* sgRNA-*fdnG*-N20	This Study
pTargetF-*pka*	*pMB1 aadA* sgRNA-*pka*-N20	This Study
pTargetF-*yfiR*	*pMB1 aadA* sgRNA-*yfiR*-N20	This Study
pTargetF-*fadB*	*pMB1 aadA* sgRNA-*fadB*-N20	This Study
pTargetF-*hofC*	*pMB1 aadA* sgRNA-*hofC*-N20	This Study
pTargetF-*yjbJ*	*pMB1 aadA* sgRNA-*yjbJ*-N20	This Study
pTargetF-*fliC*	*pMB1 aadA* sgRNA-*fliC*-N20	This Study
pTargetF-*hdeA*	*pMB1 aadA* sgRNA-*hdeA*-N20	This Study
pTargetF-*mglB*	*pMB1 aadA* sgRNA-*mglB*-N20	This Study
pTargetF-*csgA*	*pMB1 aadA* sgRNA-*csgA*-N20	This Study
pTargetF-*ssrA*	*pMB1 aadA* sgRNA-*ssrA*-N20	This Study
pTargetF-*arnA*	*pMB1 aadA* sgRNA-*arnA*-N20	This Study

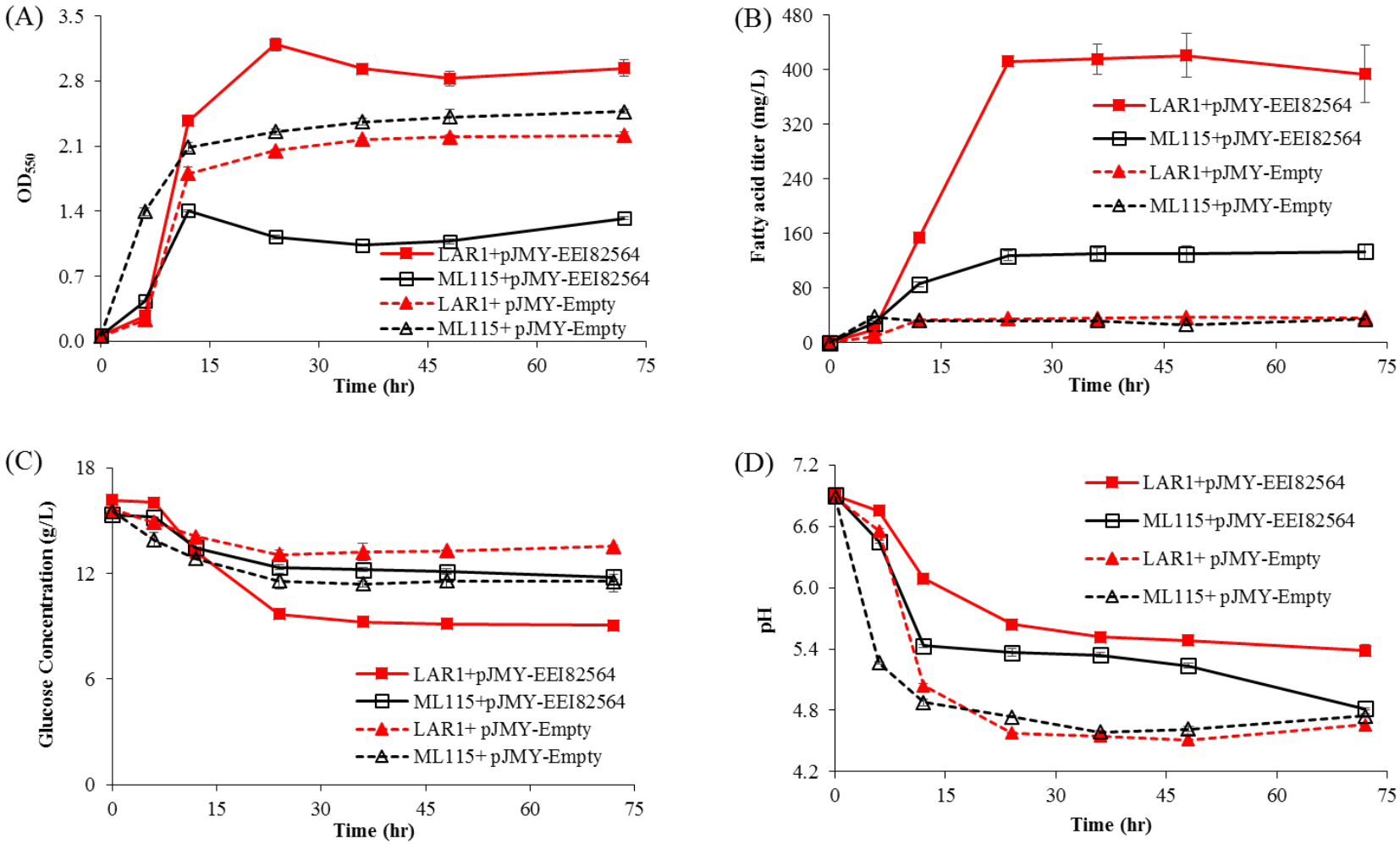
Table 3. Genes with consistent and statistically significant fold changes at three time points between LAR1+pJMY-Empty and ML115+pJMY-Empty, and between LAR1+pJMY-EEI82564 and ML115+pJMY-EEI82564, which could be affected by the mutant *rpoC* gene.
MFC: The minimal fold change is the smallest fold change in three time points.

Bnum	Name	Descriptions	MFC
b4026	*yjbE*	Uncharacterized protein YjbE	0.12
b1060	*bssS*	Biofilm regulator	0.29
b3449	*ugpQ*	Glycerophosphodiester phosphodiesterase, cytosolic	0.38
b1954	*dsrA*	Small regulatory RNA	0.40
b4376	*osmY*	Periplasmic chaperone protein	0.42
b2621	*ssrA*	tmRNA	0.43
b3631	*waaG*	Glucosyltransferase I	0.44
b2392	*mntH*	Manganese/divalent cation transporter	0.50
b1950	*fliR*	Flagellar biosynthesis protein	0.54
b1951	*rcsA*	DNA-binding transcriptional activator	0.54
b4092	*phnP*	5-phospho-alpha-D-ribosyl 1,2-cyclic phosphate phosphodiesterase	0.63
b3317	*rplB*	50S ribosomal subunit protein L2	0.67
b2937	*speB*	Agmatinase	0.70
b2604	*yfiN*	Putative membrane-anchored diguanylate cyclase with an N-terminal periplasmic domain	2.67
b1475	*fdnH*	Formate dehydrogenase-N, Fe-S (beta) subunit, nitrate-inducible	2.64
b0158	*btuF*	Vitamin B12 transporter subunit: periplasmic-binding component of ABC superfamily	2.54
b2605	*yfiB*	OM lipoprotein putative positive effector of YfiN activity	2.37
b3846	*fadB*	Fused 3-hydroxybutyryl-CoA epimerase/delta(3)-cis-delta(2)-trans-enoyl-CoA isomerase/enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	2.34
b2584	*pka*	Protein lysine acetyltransferase	2.18
b1476	*fdnI*	Formate dehydrogenase-N, cytochrome B556 (gamma) subunit, nitrate-inducible	1.96
b0107	*hofB*	T2SE secretion family protein; P-loop ATPase superfamily protein	1.92
b0159	*mtn*	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	1.91
b0106	*hofC*	Assembly protein in type IV pilin biogenesis, transmembrane protein	1.88
b3559	*glyS*	Glycine tRNA synthetase, beta subunit	1.86
b3845	*fadA*	3-ketoacyl-CoA thiolase (thiolase I)	1.70
b2235	*nrdB*	Ribonucleoside-diphosphate reductase 1, beta subunit, ferritin-like protein	1.70
b3600	*mtlD*	Mannitol-1-phosphate dehydrogenase, NAD-dependent	1.68
b2603	*yfiR*	Putative periplasmic inhibitor of YfiN activity	1.61
b1854	*pykA*	Pyruvate kinase II	1.42

Table 4. Genes with consistent and statistically significant fold changes at three time point between LAR1+pJMY-EEI82564 and ML115+pJMY-EEI82564, which could be affected by the mutant *basR* gene.
MFC: The minimal fold change is the smallest fold change in three time points.

Bnum	Name	Descriptions	BasSR	Sigma70	MFC
b4045	*yjbJ*	Stress-induced protein, UPF0337 family	3	FALSE	0.14
b4376	*osmY*	Periplasmic protein	3	TRUE	0.18
b3510	*hdeA*	Stress response protein acid-resistance protein	3	FALSE	0.29
b1950	*fliR*	Flagellar export pore protein	3	TRUE	0.35
b4113	*basR*	Response regulator in two-component regulatory system with BasS	0	FALSE	7.21
b2149	*mglA*	Fused methyl-galactoside transporter subunits of ABC superfamily: ATP-binding components	3	TRUE	5.78
b1037	*csgG*	Curli production assembly/transport outer membrane lipoprotein	1	FALSE	3.51
b1014	*putA*	Fused DNA-binding transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	1	TRUE	1.91
b0964	*yccT*	UPF0319 family protein	2	FALSE	1.78

Figures


(A)
OD550
Time (hr)
LAR1+pJMY-EEI82564
ML115+pJMY-EEI82564
LAR1+ pJMY-Empty
ML115+ pJMY-Empty
(B)
Fatty acid titer (mg/L)
Time (hr)
LAR1+pJMY-EEI82564
ML115+pJMY-EEI82564
LAR1+pJMY-Empty
ML115+pJMY-Empty
(C)
Glucose Concentration (g/L)
Time (hr)
LAR1+pJMY-EEI82564
ML115+pJMY-EEI82564
LAR1+ pJMY-Empty
ML115+ pJMY-Empty
(D)
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ML115+ pJMY-Empty

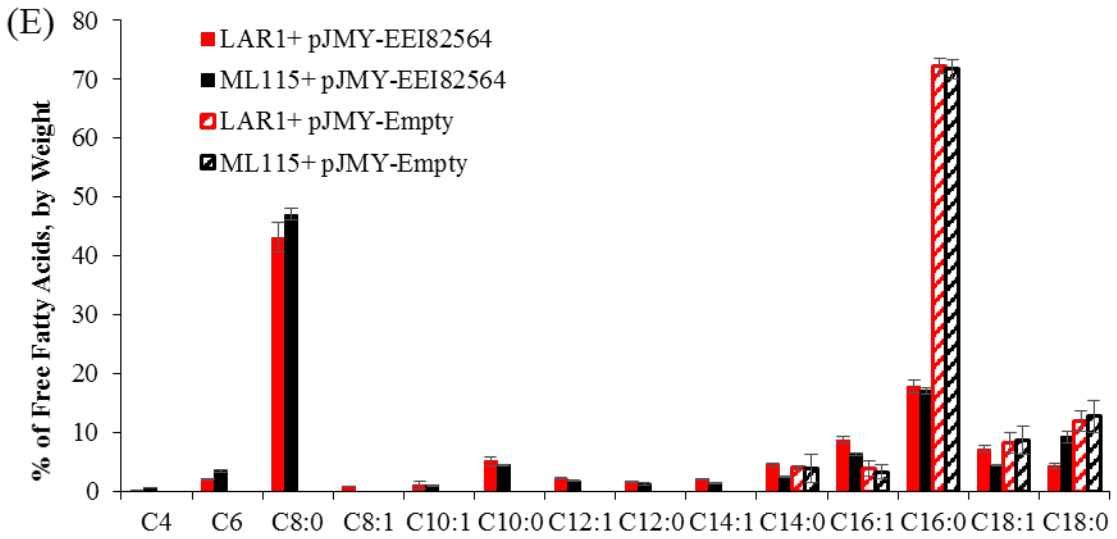

Figure 1. Strains characteristics during fatty acid production. Strains containing plasmid pJMY-EEI82564 or pJMY-Empty were grown for 72 hours. (A) Growth; (B) Total fatty acid titer (mg/L); (C) Glucose concentration in medium (g/L); (D) pH of the medium; (E) Relative distribution, by weight.
Values are the average of four biological replicates, with error bars indicating one standard deviation.

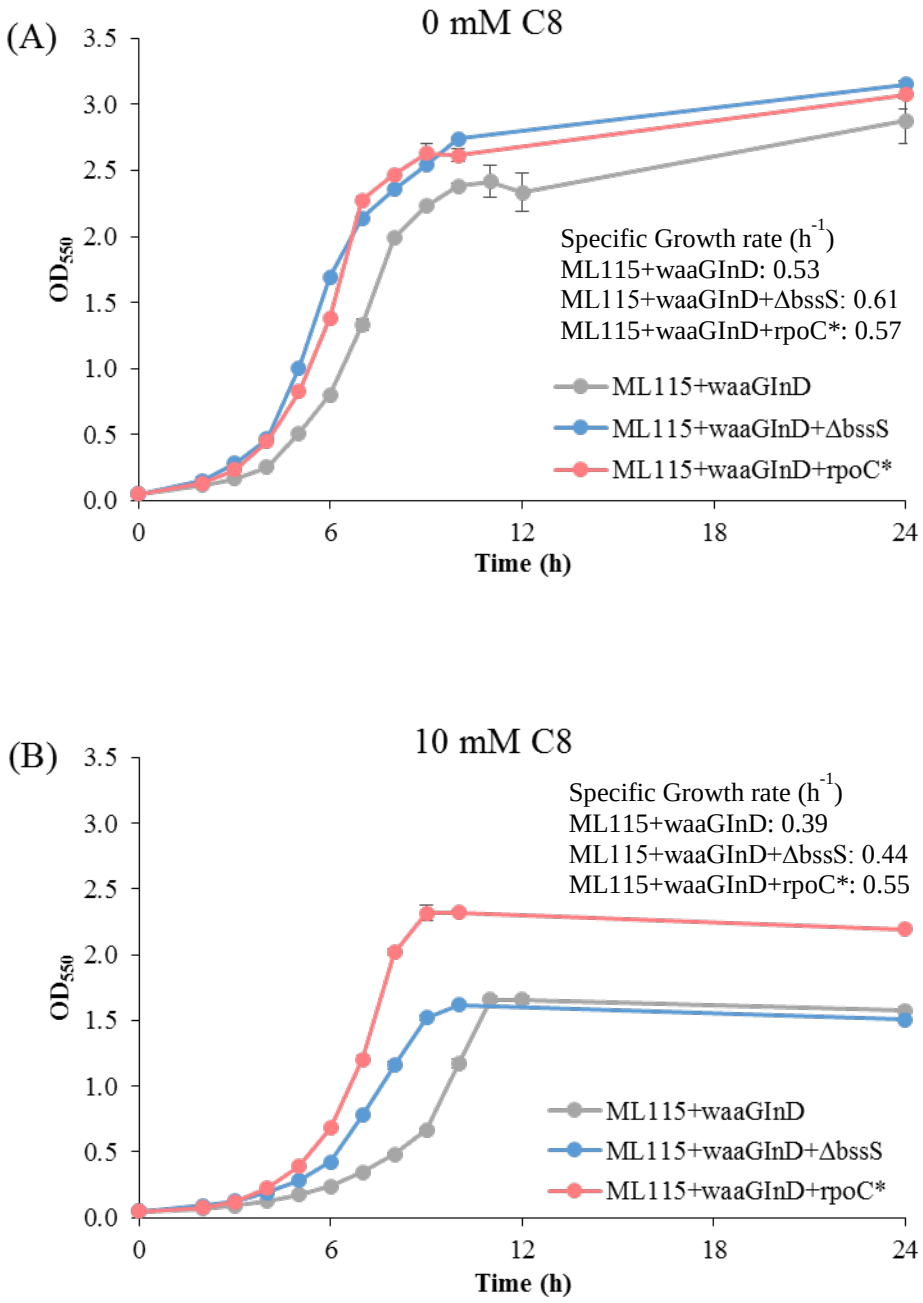

Figure 2. Knock out the *bssS* gene in ML115+waaGInD strain did effect on the growth in MOPS media with 2% glucose with 0 mM or 10 mM octanoic acid at 37 ºC. (A) Control group medium without C8; (B) Challenging group with 10 mM C8.
Values are the average of three biological replicates, with error bars indicating one standard deviation. The line of fit when estimating the specific growth rate had an R^2 value ≥0.9.

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CHAPTER 6. LESSONS IN MEMBRANE ENGINEERING FOR OCTANOIC ACID PRODUCTION FROM ENVIRONMRNTAL *ESCHERICHIA COLI* ISOLATES

Manuscript submitted to *Applied Environmental Microbiology*

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Abstract

Fermentative production of many attractive biorenewable fuels and chemicals is limited by product toxicity in the form of damage to the microbial cell membrane. Metabolic engineering of the production organism can help to mitigate this problem, but there is need for identification and prioritization of the most effective engineering targets. Here, we use a set of previously-characterized environmental *E. coli* isolates with high tolerance and production of octanoic acid, a model membrane-damaging biorenewable product, as a case study for identifying and prioritizing membrane engineering strategies. This characterization identified differences in the membrane lipid composition, fluidity, integrity and cell surface hydrophobicity relative to the lab strain MG1655. Consistent with previous publications, decreased membrane fluidity was associated with increased fatty acid production ability. Maintenance of high membrane integrity or longer membrane lipids seemed to be of less importance than fluidity. Cell surface hydrophobicity was also directly associated with fatty acid production titers, with

the strength of this association demonstrated by expression tuning of the multiple stress resistance outer membrane protein BhsA. Expression tuning of *bhsA* was effective in altering hydrophobicity, but the direction and magnitude of the change differed between strains. Thus, additional strategies are needed to reliably engineer cell surface hydrophobicity. This work demonstrates the ability of environmental microbiological studies to impact the metabolic engineering design, build, test, learn cycle and possibly increase the economic viability of fermentative bioprocesses.

Importance

Production of bulk fuels and chemicals in a bio-based fermentation process requires high product titers. This is often difficult to achieve, because many of the target molecules damage the membrane of the microbial cell factory. Engineering the composition of the membrane in order to decrease its vulnerability to this damage has proven to be an effective strategy for improving bio-production, but additional strategies and engineering targets are needed. Here, we study a small set of environmental *Escherichia coli* isolates that have higher production titers of octanoic acid, a model biorenewable chemical, relative to the lab strain MG1655. We found that membrane fluidity and cell surface hydrophobicity are strongly associated with improved octanoic acid production. Fewer genetic modification strategies have been demonstrated for tuning hydrophobicity relative to fluidity, leading to the conclusion that there is a need for expanding hydrophobicity engineering strategies in *E. coli*.

Introduction

Throughout the vast majority of human history, transportation energy and commodity goods have been obtained from woody biomass, crops, and animals (1–3). However, in the past century, petroleum has become the predominant source of carbon and energy for the production

fuels and chemicals, with a variety of undesirable outcomes (4–6). The fermentative production of bulk fuels and chemicals, such as ethanol, lactic acid and the ABE process, for example, were commercially viable prior to establishment of the petrochemical industry (7, 8). However, the economically viable production of bio-based fuels and chemicals that could displace petroleum-derived molecules still remains challenging (6).

One of the limitations of fermentative production of bulk fuels and chemicals is toxicity, both in terms of the product molecules and the biomass-derived carbon streams (9, 10). This toxicity can be addressed through *in-situ* product removal (11–14) and/or increasing the robustness of the microbial biocatalyst (15–18). Design strategies for robust biocatalysts can be based on known mechanisms of inhibition and tolerance (19–21). Implementation of the "learn" step of the design, build, test, learn cycle (22, 23) can provide inspiration for such rational design strategies. This learning can come from characterization of evolved strains (21) or wild populations (24).

Fatty acids have a large and increasing industrial demand due to their wide range of applications. Short-chain fatty acids are directly used as a food preservative (25, 26) and dietary supplement (27). Fatty acids also can be used as precursors for a variety of industrial chemicals, such as alkanes (28, 29), α-olefins (30, 31), and fatty acid methyl or ethyl esters (32). Extensive work has been done to improve the fermentative production of fatty acids (5, 33–36). However, as with many other desirable biorenewable products (37, 38), fatty acid toxicity to microbes (9, 10, 19, 39) limits the product titers, and thus negatively impacts the economic viability of bio-based fatty acid production. Substantial progress has been made in addressing cytotoxicity of fatty acids in *E. coli* (9, 18, 33), with a variety of studies concluding that membrane damage is the major mechanism of fatty acid toxicity (9, 10, 18, 19, 39). This membrane damage

corresponds to a decrease in membrane integrity (9) and perturbation of membrane fluidity (9, 18). Engineering the cell membrane composition (e.g. lipid distribution) to mitigate the damage imposed by fatty acids (33, 40), enabling the production of trans unsaturated fatty acids (16), and altering of the phospholipid head distribution (41), have proven effective for increasing fatty acid tolerance and/or production. Despite this progress, further increases in fatty acid titer are desirable - particularly in terms of short-chain fatty acids, such as octanoic acid (C8).

A previously-described collection of environmental *E. coli* isolates was found to have wide variation in membrane properties such as the hydrophobicity and the zeta potential (42). Here, we characterized these strains for their ability to tolerate and produce octanoic acid. This characterization identified membrane properties that are promising additions to the arsenal of strategies for improving microbial robustness for increasing production of biorenewable fuels and chemicals.

Materials and Methods

Strains, plasmids and bacterial cultivation

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown overnight in 250 mL flasks with 10 mL of MOPS media with 2.0 wt% dextrose at pH 7.00±0.05, at 37 ºC and 250 rpm. For octanoic acid tolerance tests, the overnight seed culture was diluted to an optical density at 550 nm (OD_{550}) of 0.05. For fatty acid fermentation, measurement of membrane leakage, membrane fluidity, cell hydrophobicity, and cell membrane composition, the overnight seed culture was diluted to an OD_{550} of 0.1. Ampicillin (100 mg/L), and kanamycin (50 mg/L), were added as needed. All growth media and phosphate buffered saline solutions had an initial pH of 7±0.05.

Octanoic acid tolerance test

Tolerance to octanoic acid was assessed in 250 mL baffled flasks with 25 mL MOPS medium with 2.0 wt% dextrose with or without exogenous 10 mM C8, at 37 ºC and 200 rpm. Octanoic acid was provided via a stock solution containing 4.0 M C8 in ethanol. The pH of the media was adjusted using 2.0 M KOH.

Fatty acid production

Strains were transformed with the pJMY-EEI82564 plasmid (18) by electroporation and grown on LB plates with ampicillin 30 ºC overnight. Individual colonies were inoculated in 10 mL MOPS media with 2.0 wt% dextrose and ampicillin in 250 mL flasks at 30 ºC on a rotary shaker at 250 rpm overnight. These seed cultures were subsequently inoculated into 250 mL flasks containing 50 mL of MOPS media with 2.0 wt% dextrose, ampicillin and 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an initial OD_{550} of 0.1. The flasks were incubated in a rotary shaker at 200 rpm and 30 ºC.

Determination of fatty acid titers

Fatty acid production was quantified by fatty acid extraction from samples containing both media and cells. The extracted fatty acids were derivatized and measured at the ISU W.M. Keck Metabolomics Research Laboratory via an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectroscope (GC-MS), as previously described (63). Briefly, 1 mL culture was transferred into a 2 mL microcentrifuge tube and 125 µL 10% NaCl (w/v), 125 µL acetic acid, 20 µL internal standard (1 µg/ µL C7, C11, C15 in EtOH), and 500 µL ethyl acetate were added sequentially. The mixture was vortexed for 30s and centrifuged at 16,000×g at room temperature for 10 min. 250 µL of the top layer was transferred into a glass tube and 2.25 mL 30:1 EtOH: 37% HCl (v/v) was added. This mixture was then incubated at 55 ºC for 1 hour, then

cooled to room temperature. Then, 1.25 mL each of ddH_2O and hexane were added, followed by vortexing and centrifugation at 2,000×g for 2 min. The top hexane layer was then analyzed by GC-MS using the following program: the initial temperature was 50 ºC, hold for 1 min, with the following temperature ramp: 20 ºC/min to 140 ºC, 4 ºC/min to 220 ºC, and 5 ºC/min to 280 ºC with 1 ml/min helium carrier gas.

The Enhanced Data Analysis (Agilent Technologies) and NIST 17 Mass Spectral Library software were used for peak identification. The relative retention factors of C7, C11, and C15 were used to calculate the relative amounts of the individual fatty acids analyzed.

Membrane lipid composition

Seed cultures were inoculated into 250 mL baffled flasks with 50 mL MOPS medium with 2.0 wt% dextrose, at 30 ºC and 250 rpm. For analysis of C8 challenge, *E. coli* cells were harvested at mid-log (OD~0.8), resuspended in 25 mL media with 0 mM or 30 mM C8 at pH 7.0, and incubated for 3 hours at 30 ºC. For analysis of C8 production, cells were harvested at 24 and 48 hours.

Harvested cells were washed twice in cold sterile water, resuspended into 6 mL methanol, and sonicated for three 30 s bursts. Then, 1.4 mL of this sonicated solution was transferred into each of three glass tubes, to be processed in parallel (64). Twenty µL of a solution containing 1 µg/µL each of C7, C11, and C15 in methanol was added. These mixtures were incubated at 70 ºC for 15 min and cooled to room temperature. The cells were centrifuged at 4,000×g for 5 min, and the resulting supernatant and pellet were treated separately. The supernatant was transferred into a new glass tube with 1.4 mL nanopure water and vortexed. 750 µL of chloroform was added to the pellet, followed by vortexing and shaking in a horizontal shaker at 150 rpm, 37 ºC for 5 min. The diluted supernatant was added back to the chloroform-

treated pellet. This mixture was vortexed for 2 min, then centrifuged at 3,000×g for 5min. The bottom layer was transferred to a new glass tube and all solvent removed with an N-Evap nitrogen tree evaporator. Two mL of 1.0N HCl in methanol was added to the dried samples, heated at 80 ºC for 30 min, then cooled to room temperature. Two mL 0.9 wt% aqueous NaCl and 1.0 mL hexane was added, followed by vortexing for 2 min and centrifugation at 2,000×g for 2 min. The upper layer was analyzed by GC, using the method described above.

The weight-average lipid length was calculated as previously described (9). The percent abundance of unsaturated fatty acids was calculated as the sum of the percent abundance of C12:1, C14:1, C16:1, and C18:1.

Membrane permeability

Cells were harvested at 24 h and 48 h. Following centrifugation at 4,500×g and 4 ºC for 10 min, cell pellets were washed twice with PBS, and resuspended in PBS at a final OD_{550} ~ 1. Then, 100 µL of resuspended cells were diluted with 900 µL PBS. Cells were stained with 1 µL of 5 mM SYTOX Green in dimethyl sulfoxide (Invitrogen, Carlsbad, CA), for a final concentration of 5 mM. Cells were then analyzed by flow cytometry at the ISU Flow Cytometry Facility on a BD Biosciences FACSCanto II (18). About 18,000 events were tested per sample, and each sample had three parallel groups.

Membrane fluidity

Membrane polarization was measured using 1,6-diphenyl-1,3,5-hexatirene (DPH) as previously described (9, 47). Harvested cells were prepared as described above for assessment of membrane permeability. Five hundred µL of cells in PBS at an OD_{550} of ~ 1 were transferred to a 1.5 mL centrifuge tube containing 500 µL of 0.4 µM DPH in PBS. The mixture was vortexed and incubated at 37 ºC in the dark for 30 min. The samples were then centrifuged (5,000×g, 5

min) and the cell pellets were resuspended in 500 µL PBS. One hundred µL of this mixture was transferred into sterile black-bottom Nunclon delta surface 96-well plates with 4 replicates. A suspension of cells with no DPH was used as a control. Membrane fluorescence polarization values were determined based on vertical and horizontal fluorescence readings, which were assessed by via the BioTek Synergy 2 Multi-Mode microplate reader at the ISU W.M. Keck Metabolomics Research Laboratory.

Cell surface hydrophobicity

The method for measuring cell surface hydrophobicity was based on the previously described procedure (42, 65, 66). The seed culture was inoculated into 25 mL MOPS media with 2 wt% dextrose with or without exogenous 10 mM C8, at 30 ºC with rotary shaking at 250 rpm. Cells were harvested at mid-log phase (OD_{550}~0.8) followed by centrifugation at 4,500×g and room temperature for 10 min. Cell pellets were then washed twice with PBS buffer, and resuspended in PBS at a final of OD_{550}~0.6. Four mL of cell suspension was added to a glass tube, and the OD_{550} was measured (OD_1). Then, 1.0 mL of dodecane was added, and the mixture was mixed using a multi-tube vortexer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 2500 rpm for 10 minutes. The mixtures were left to settle for 15 min at room temperature and the OD_{550} of the aqueous phase (OD_2) was measured. Partitioning of the bacteria suspension was calculated using the following equation:

Percent partitioning = $[(OD_1-OD_2)/OD_1]*100$

Results

Environmental isolates show increased fatty acid tolerance and production

It has previously been demonstrated that short chain fatty acids are inhibitory to *E. coli*, having a detrimental impact on both growth and on fatty acid production (9, 18, 43). Several *E.*

coli strains were previously isolated from Squaw Creek in Ames, IA, and characterized in terms of various cell surface properties and phylotype (42). Given that these environmental isolates have previously demonstrated an ability to survive and propagate in harsh environmental conditions, we proposed that these environmental isolates may be able to provide insight into design strategies for developing robust microbial cell factories for short-chain fatty acid production.

Fifteen of these previously-characterized environmental isolates were compared to the standard K-12 lab strain MG1655 in terms of short-chain fatty acid sensitivity and production (Table 2). Specifically, strains were grown in MOPS media with 2.0 wt% glucose at 37 ºC with and without 10 mM C8 at pH 7.0. Nearly all of the strains showed less than 20% inhibition relative to growth in the absence of C8. Note that environmental isolate #6 showed a very high C8 sensitivity, with approximately 90% inhibition. All of the environmental isolates, except for strains #48 and #236, showed significantly ($p < 0.05$) less inhibition by 10 mM C8 relative to MG1655 (Table 2).

Given these promising results regarding fatty acid tolerance, we then characterized short-chain fatty acid production for a smaller set of these environmental isolates via expression of the *A. tetradius* thioesterase. This set was selected so that there was at least one isolate from each each of the phylogroups. Fatty acid production was characterized for seven of these environmental isolates and MG1655 in MOPS media with 2.0 wt% glucose at 30 ºC (Table 2). All of the environmental isolates had significantly higher fatty acid titers than MG1655. Specifically, MG1655 produced 56.4±0.8 mg/L while all of the isolates produced at least 145 mg/L. All of the environmental isolates also showed an approximately 5-fold increase in biomass production during fatty acid production relative to MG1655. These results provide further

motivation for characterizing these isolates in order to identify design strategies for increasing fatty acid production in industrial strains.

We selected three environmental isolates for further characterization. Isolate #18 had the lowest fatty acid titer (148±3 mg/L) while isolates #83 and #292 had the highest fatty acid titers (251 mg/L). Isolate #292 also had the smallest OD_{550} relative to the other environmental isolates.

Measurement of the free fatty acid chain length distribution indicates that the wild-type lab strain MG1655 shows slightly improved specificity for C8:0 fatty acids relative to the environmental isolates (Figure 1). Specifically, the pool of free fatty acids produced by MG1655 contained more than 75 wt% C8. The free fatty acid pools produced by the environmental isolates contained between 50 and 60 wt% C8 with a shifting of the distribution towards chain lengths of 14 or more carbons.

Identification of design strategies from the membrane lipid distribution

Damage to the microbial membrane is a key component of fatty acid toxicity (9, 19, 39) and changes to the membrane composition have been associated with increased fatty acid tolerance and production (16, 18, 33, 40, 41). Characterization of the membrane of each of our selected focal *E. coli* strains during fatty acid challenge and during fatty acid production supports the contribution of the lipid tail distribution to differences in strain robustness (Table 3).

A few differences in membrane lipid tail distribution were observed between the environmental isolates and MG1655 regardless of the presence or production of octanoic acid (Table 3). Specifically, in all conditions strain #83 had above-average abundance of C12:0, while MG1655 had below-average abundance. Also, each of the environmental isolates showed increased abundance of C12:1 during fatty acid production relative to the non-production condition. This trend was not observed for MG1655.

It has previously been demonstrated that increasing the average fatty acid chain length in the membrane can improve fatty acid production (40). Each of the environmental isolates characterized here showed a decrease in average lipid length during fatty acid production relative to the non-production condition (Table 3). While MG1655 showed a similar decrease, the magnitude was smaller.

The relative degree of unsaturation is also known to be a key metric of membrane fatty acid composition (9, 18, 33). Previous reports have described an increase in the relative abundance of unsaturated C_{16} and C_{18} lipids in the membrane during fatty acid production by a K-12 *E. coli* strain (33). However, the characterization reported here observed a significant decrease in the relative abundance of unsaturated fatty acids in the membrane of MG1655 during fatty acid production (Table 3). The environmental isolates also showed, in general, a decrease in the abundance of unsaturated lipids during fatty acid production. However, in MG1655 the abundance of unsaturated membrane lipids was much smaller during fatty acid production than for the environmental isolates.

The opposite trend was observed for the relative abundance of cyclic fatty acids: their abundance generally increased in all strains during fatty acid production. A broad interpretation of these trends is that changes in the MG1655 membrane during fatty acid production mainly consisted of changes in the abundance of unsaturated fatty acids. However, during fatty acid production the membranes of the environmental isolates changed in terms of length, not unsaturation.

In addition to the membrane composition metrics described above, the C16:1/C16:0 ratio also seems to be useful for comparing these strains (Figure 2). The MG1655 membrane showed a significant decrease in this ratio during challenge with 30 mM C8 relative to the untreated

control, decreasing from 0.32 to 0.23. In contrast, each of the environmental isolates showed an increase in this ratio. For example, for strain #18 this ratio increased from 0.46 to 0.63 ($p < 0.0006$). During fatty acid production, the three environmental isolates all had a significantly higher C16:1/C16:0 ratio than the MG1655 strain ($p < 0.0006$). At 24 hours, this ratio was 0.07 for MG1655, but was 0.20 for strain #18 and 0.37 for strain #292.

Previous membrane engineering efforts have aimed to engineer the membrane composition in terms of average lipid length and abundance of unsaturated lipids. Our results suggest that it may also be useful to target the C16:1/C16:0 ratio.

Decreased membrane fluidity is associated with increased fatty acid production

Membrane damage is generally considered a key mechanism of *E. coli* inhibition during challenge with or production of short chain fatty acids, along with many other biorenewable fuels and chemicals (9, 16, 18). The membrane composition can be quantified by metrics such as the lipid tail distribution, as described above. However, the membrane also consists of many proteins, sugars, and phospholipid head groups. Metrics such as membrane fluidity and integrity reflect the overall function and state of the membrane.

Maintenance of appropriate membrane fluidity is vital to membrane function. In general, membrane fluidity trends with the relative abundance of unsaturated lipids (44), though other membrane components can also contribute to this property (16, 45, 46). Membrane fluidity was measured via cell membrane polarization, where increased membrane polarization corresponds to decreased membrane fluidity (9, 47). Our previous studies have shown that the membrane polarization of MG1655 decreased substantially during challenge with exogenous C8 without prior adaptation (9). However, cells that had 3 hours of prior exposure to 30 mM C8 were able to maintain a membrane polarization of approximately 0.3 during exogenous C8 challenge. These

prior results demonstrate that free fatty acids could change membrane polarization, but changes in the membrane composition could make the cell membrane more resistant to this effect.

Membrane polarization was assessed for the fatty acid producing strains at 24 and 48 hours (Figure 3A). At 24 hours, isolates #18 and #292 had significantly ($p<0.05$) increased polarization relative to MG1655. At 48 hours, all three isolates had significantly increased polarization relative to MG1655. The increased polarization during fatty acid production for the environmental isolates relative to MG1655, indicates that the isolates have a lower fluidity than MG1655.

Previous studies of an evolved *E. coli* strain and an *E. coli* strain engineered to produce a novel membrane lipid tail also reported that increased membrane polarization was associated with increased fatty acid production (9, 16). The isolate characterization described here provides further evidence that decreasing membrane fluidity is a promising strategy for improving short chain fatty acid tolerance and production. Surprisingly, the isolates all had a higher abundance of unsaturated lipids relative to MG1655, leading to the expectation of a higher fluidity. Thus, differences in fluidity cannot be solely attributed to the relative abundance of unsaturated fatty acids in the membrane.

The isolates also differ from MG1655 in that their polarization value significantly ($p < 0.05$) increased from 24 hours to 48 hours (Figure 3A). These results indicate that the environmental isolates are able to further modulate their membrane fluidity. For isolates #83 and 292, this further increase in polarization is surprising, given the observed significant ($p < 0.05$) increase in the percentage of unsaturated fatty acids (Table 3) and the C16:1/C16:0 ratio (Figure 2). This provides further demonstration that fluidity is not solely a function of unsaturated fatty acid content. Isolate #18 did not have a significant increase in the percentage of unsaturated fatty

acids or C16:1/C16:0 ratio. Instead, isolate #18 showed a significant ($p=0.041$) increase in the average lipid length. Thus, the environmental isolates might be using distinct strategies to control membrane fluidity during fatty acid production.

Investigation of membrane integrity during fatty acid production

Decreased membrane integrity is a widespread problem during microbial production of fuels and chemicals (15, 16, 18, 39, 41). Membrane integrity can be quantified via the permeability of cells to the SYTOX nucleic acid dye (33). At both time points during fatty acid production, MG1655 had dramatically decreased SYTOX permeability relative to the environmental isolates (Figure 3B). This could be due to the very low fatty acid titer of MG1655 (Table 2). A large percentage of cells from the three environmental isolates were SYTOX-permeable. Specifically, all of the strains were more than 99% permeable at 48 hours. These results show that the environmental isolates have decreased cell membrane integrity relative to MG1655 during fermentation, which indicates that the lower fatty acid titer achieved by MG1655 cannot be attributed to a loss of membrane integrity.

Higher cell surface hydrophobicity leads to higher fatty acid production

The measurements of membrane polarization and integrity described above reflect the physical state of the phospholipid bilayer. Contrastingly, measurement of the cell surface hydrophobicity reflects the abundance and type of proteins and sugars on the cell surface (ref). The environmental isolates used in this study are part of a larger set that was previously characterized in terms of various cell surface properties, including hydrophobicity (42). Hydrophobicity was found to range from 1 to 90 % adhesion to hydrocarbons (42). Thus, we also characterized the cell surface hydrophobicity during fatty acid challenge in the current work.

The three focal environmental isolates all had increased hydrophobicity relative to MG1655, with and without exogenous C8 challenge in MOPS minimal media at 30 ºC (Figure 4). Also, each of the environmental isolates showed a decrease in hydrophobicity during C8 challenge relative to the control condition. Thus, the environmental isolates differ from MG1655 in both the value of the hydrophobicity and its modulation in response to C8.

A positive correlation between the cell surface hydrophobicity and fatty acid production was observed (Figure 4). Specifically, fatty acid titers increased as cell hydrophobicity increased. These results suggest that increasing the cell surface hydrophobicity might be a strategy for improving fatty acid production. It has been previously demonstrated in yeast that increasing the cell surface hydrophobicity increased tolerance of nonane (48) and increased production of ethanol in the presence of inhibitors such as carboxylic acids, furfural and biomass hydrolysate (49).

Expression tuning of outer membrane protein BhsA in *E. coli* was previously demonstrated to change the cell surface hydrophobicity (50). Here, we altered the cell surface hydrophobicity of the four focal strains by expressing *bhsA* via the pACYC184 plasmid with the MG1655 *bhsA* promoter (15). For strain MG1655 and isolate 18, the cell surface hydrophobicity significantly ($p < 0.05$) increased when *bhsA* expression was increased in either the absence or the presence of C8 (Figure 5). Expression of *bhsA* had no impact on the hydrophobicity of strain #83 in the absence of C8, but decreased the hydrophobicity in the presence of C8. In contrast, for isolate #292, *bhsA* expression decreased hydrophobicity only in the absence of C8. Thus, in accordance with previous reports, expression tuning of *bhsA* can alter the cell surface hydrophobicity of *E. coli* strains. However, the trends were not consistent across strains and conditions.

This perturbation of the hydrophobicity was performed in order to investigate the relationship between cell surface hydrophobicity and fatty acid production. Consistent with the increase in hydrophobicity for MG1655+*bhsA* and #18+*bhsA*, fatty acid titers also significantly increased (Figure 5B). The association of hydrophobicity and fatty acid production was conserved for the isolates #83 and 292. Specifically, these strains each showed a decrease in cell surface hydrophobicity, either in the presence or absence of C8, and they each showed a significant decrease in fatty acid titer. Thus, tuning of cell surface hydrophobicity is a promising method of improving fatty acid production, but additional genetic targets for altering hydrophobicity in *E. coli* are needed.

Discussion

Many physical and genetic factors determine microbial tolerance to and production of inhibitory chemicals. Implementation of the design, build, test, learn cycle (22) in the construction of improved fermentation organisms, particularly in regards to membrane engineering, can benefit from the characterization of evolved or non-model organisms (18, 20, 51–53). The microbial cell membrane is an increasingly important engineering target for the design of high-performing microbial biocatalysts (54, 55). In this case, we found that several aspects of the *E. coli* membrane, such as composition and physical properties, were associated with increased tolerance to and production of octanoic acid, a representative fatty acid.

The ability to modulate the relative abundance of unsaturated lipids and the resulting impact on tolerance phenotypes has been previously reported in *E. coli* and *S. cerevisiae* (33, 44, 56). Here, we saw that the high-producing environmental isolates did have an altered abundance of unsaturated lipids relative to MG1655 (Table 3). Additionally, our results suggest that the C16:1/C16:0 ratio may be a useful metric distinct from the standard consideration of total pools

of saturated and unsaturated lipids. However, the observed differences in membrane fluidity did not appear to be solely attributable to the relative abundance of unsaturated lipids (Figure 3A).

Engineering of the average lipid length has also been previously reported as strategy for increasing fatty acid tolerance and production in *E. coli* (40), and an evolved E. coli strain with increased fatty acid tolerance and production also showed an increase in average lipid length (18). Each of environmental isolates characterized here showed a decrease in the average lipid length during fatty acid production relative to the control condition (Table 3). Thus, an increase in average lipid length is not required for attainment of the high fatty acid titers achieved by these isolates, possibly due to other differences in membrane composition.

The isolates characterized here show intriguing changes in the relative abundance of these cyclopropane fatty acids (Table 3). The relative abundance of these molecules has been previously reported as impacting E. coli tolerance of various stressors, such as heat, pressure and acid stress (57, 58). However, previous attempts to increase the abundance of these fatty acids in order to increase tolerance of fatty acids have not been successful (9, 10).

The overall membrane composition, not just in terms of the fatty acid tails, but other components such as proteins, phospholipid head groups and polysaccharides, contribute to the bulk membrane properties, such as fluidity, integrity, rigidity, curvature, and thickness (59–62). Our characterization of environmental isolates with increased fatty acid production titers supports the idea that membrane fluidity is a critical determinant of fatty acid production capability (Figure 3A), consistent with previous reports that decreased fluidity is associated with increased production (16, 18). However, the substantial loss of membrane integrity during the course of fatty acid production by the environmental isolates (Figure 3B) does not appear to be detrimental to their ability to achieve high fatty acid titers. This suggests that focusing on

engineering of membrane fluidity may be more impactful than trying to combat the decrease of membrane integrity often observed during bio-production.

Beyond modulation of the physical properties of the membrane lipid bilayer, the lipids, proteins and sugars associated with the membrane contribute to cell surface properties such as hydrophobicity and charge (42, 61, 62). Our characterization of these environmental isolates indicates that cell surface hydrophobicity is strongly associated with the ability to produce fatty acids at a high titer (Figure 4). Thus, engineering strategies that enable tuning of cell surface hydrophobicity may be useful for improving bio-production. Consistent with previous reports (50), expression tuning of BhsA was shown to impact cell surface hydrophobicity (Figure 5A). These changes in cell surface hydrophobicity were consistent with changes in fatty acid titers (Figure 5B). However, the impact of BhsA expression tuning on cell surface hydrophobicity varied according to the strain background, and in some cases the change was relatively small. Thus, additional strategies for predictably and substantially changing cell surface hydrophobicity could be useful for the strain design toolkit.

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Tables

Table 1. Strains and Plasmids used in this study

Strains	**Genotype or description**	**Phylogroup**	**Reference**
MG1655	*F- lambda- ilvG- rfb-50 rph-1*	A	Wild-type
#9, 18, 284	Environmental *E. coli* isolate from Squaw Creek, Ames, IA	A/C	(42)
#8, 13, 28, 48, 57, 236, 248		B1	
#6, 32, 44, 83		D/E	
#292		Unknown	
Plasmids	**Genotype or description**		
pJMYEEI82564	pTrc-EEI82564 thioesterase (TE10) from *Anaerococcus tetradius*, Amp^R		(18)
PACYC184-Kan-*ycfR*	pACYC184-Kan harboring *ycfR*, Kan^R		This work

Table 2. Environmental isolates have decreased fatty acid sensitivity and increased fatty acid production relative to MG1655. Strains were grown in MOPS minimal medium with 2.0 wt% glucose, with or without 10 mM C8 at pH 7.00±0.05. For fatty acid production, strains expressed the TE10 thioesterase from *Anaerococcus tetradius* via the pJMYEEI82564 plasmid induced with 1.0 mM IPTG. Growth was assessed at 37 ºC and production was assessed at 30 ºC.
$^{+}$indicates environmental isolates whose C8 sensitivity was not significantly different (p>0.05) from MG1655.
* indicates significantly ($p \leq 0.0001$) different fatty acid titer or OD_{550} relative to MG1655

Strain	**OD_{550} at 8 h, 37 ^{o}C**		**% decrease in OD_{550}**	**Fatty acid titer (mg/L)**		**OD_{550} during fatty acid production**	
	0 mM C8	**10 mM C8**		**24 h**	**48 h**	**24 h**	**48 h**
MG1655	2.0±0.1	1.69±0.01	15.5±0.8	52.7±0.8	56.4±0.8	0.59±0.01	0.58±0.01
#6	1.7±0.1	0.145±0.03	90±20				
#8	1.94±0.01	1.70±0.02	12.4±0.2	235±7*	213±5*	3.7±0.2*	3.92±0.05*
#9	1.8±0.0	1.69±0.02	6.1±0.1				
#13	1.8±0.1	1.7±0.0	5.6±0.3				
#18	1.84±0.02	1.67±0.02	9.2±0.2	144±2*	148±3*	3.00±0.02*	3.64±0.06*
#28	1.80±0.04	1.69±0.01	6.1±0.1				
#32	1.86±0.07	1.640±0.002	11.8±0.5				
#44	1.83±0.05	1.61±0.04	12.0±0.4	101±1*	200±10*	3.8±0.1*	4.0±0.1*
#48	2.07±0.02	1.77±0.01	14.5±0.2^{+}	200±20*	228±9*	3.6±0.1*	4.01±0.02*
#57	1.68±0.05	1.56±0.01	7.1±0.2				
#83	1.86±0.05	1.62±0.06	12.9±0.6	245±5*	251±3*	3.6±0.1*	3.98±0.01*
#236	2.04±0.01	1.72±0.01	15.7±0.1^{+}	220±10*	216±6*	3.7±0.1*	3.8±0.1*
#248	1.92±0.06	1.75±0.05	8.9±0.4				
#284	1.90±0.06	1.71±0.02	10.0±0.3				
#292	1.96±0.03	1.73±0.07	11.7±0.5	245±5*	251±2*	2.98±0.03*	2.84±0.04*

Table 3. Membrane fatty acid tail composition (mol%) differs among MG1655 and the environmental isolates during C8 challenge and fatty acid production. Strains were assessed in MOPS+2.0 wt% dextrose at 30 ºC in shake flasks at 200 rpm. Cells assessed for the control and +30 mM conditions do not encode the thioesterase. All cultures had an initial pH of 7.0. For the control and +30 mM experiments, cells were harvested at mid log, corresponding to an OD_{550} of approximately 1.0.
Numerical values are the average of three biological replicates, with the number of significant digits shown being selected based on the value of the standard deviation. Shading indicates the value relative to the median for each row according to the 95% confidence intervals. Values shaded in red are smaller than the median, values shaded in green are larger than the median.

	MG1655				#18				#83				#292			
	control	+30 mM	24 hr	48 hr	control	+30 mM	24 hr	48 hr	control	+30 mM	24 hr	48 hr	control	+30 mM	24 hr	48 hr
C12:0	0.5	1.7	0	0	0	7	3.28	2.4	3.9	4.3	4	3.97	0.3	1.03	4.02	4.2
C12:1	0	0	0	0	0	0	0.8	0.7	0	0	2.1	2.56	0	0	1.6	1.79
C14:0	4.6	3.9	3.9	5	6	4.9	11.1	9.5	12	7.1	11.2	10.7	4.3	4.2	9.4	8.9
C14:1	0	0	0	2.7	0	0	5.8	5.4	0	0	6.27	7	0	0	6.5	7.01
C16:0	47	49	54	55	46	37.3	37.4	37.3	37	39.1	33.9	30.5	42	39.6	33.4	30.6
C16:1	15.5	11.1	3.9	4.6	20.9	23.3	7.8	7.26	11.6	17	11.9	13.2	24.7	26.9	12.35	13.4
C17cyc	13.4	11	27	19.2	7	5.3	20.5	22.1	14.4	10.1	16.5	16.1	8.2	5	19	18.9
C18:0	1.11	2.2	2	2.9	2.8	3.82	1.1	2.8	0.8	2.6	2.113	2.4	0.94	1.852	1.8	2.06
C18:1	16	20	5.8	7	17.5	17.8	9.2	9.1	18.9	19	9.9	11.4	19.6	21.1	10	10.97
C19cyc	1.9	1.8	2.1	2.1	0	0.5	2.4	3.1	1.8	0.85	2	2.3	0.6	0.4	1.7741	1.91
average length	16.27	16.3	16.1	16.1	16.3	16.1	15.77	15.9	16.04	16.14	15.6	15.708	16.33	16.343	15.73	15.73
% unsaturated	31	30	10	14	39	41.4	23.6	22.7	30.4	35.9	30.2	34.1	44.4	48.2	31	33.5
% cyclic	15.4	12.1	30	20.2	7	5.8	23	24.6	16.3	10.9	18.5	18.4	8.8	5	20.8	20.8

Figures

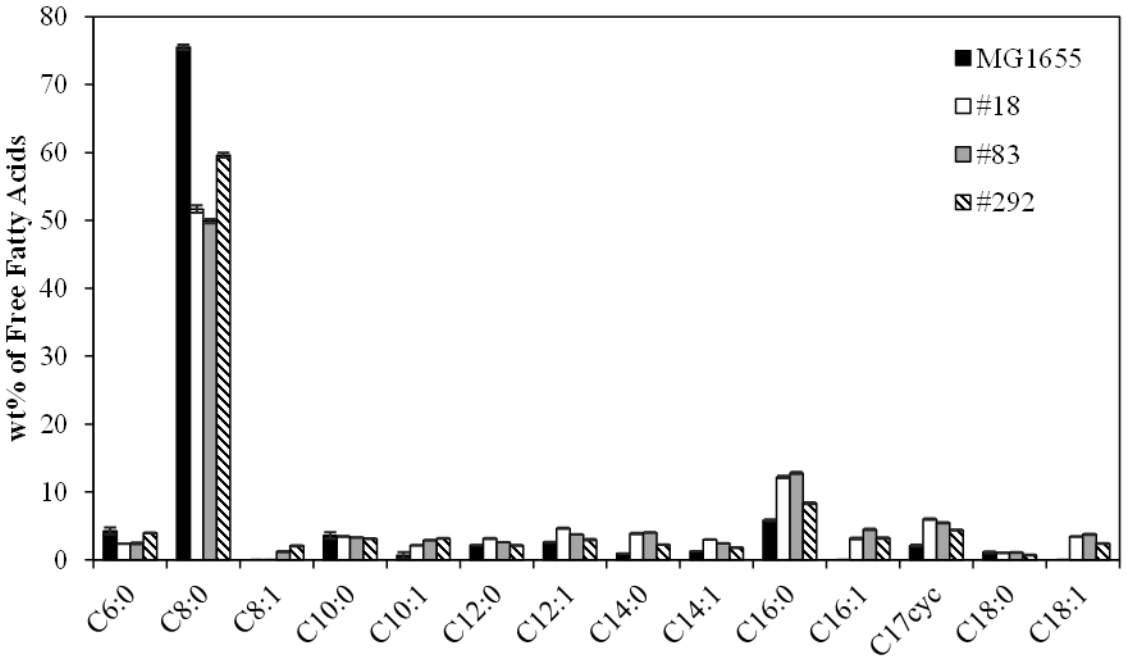

Figure 1. The focal environmental *E. coli* isolates have decreased chain-length specificity among the free fatty acid pool relative to MG1655. All strains contain the pJMYEEI82564 plasmid encoding the *A. tetradius* thioesterase.
Strains were grown for 48 hours in MOPS mineral salts medium with 2.0% (w/v) dextrose at 30 °C, initial pH 7.0, 200 rpm with 1.0 mM IPTG. Values are the average of three biological replicates with error bars indicating one standard deviation.

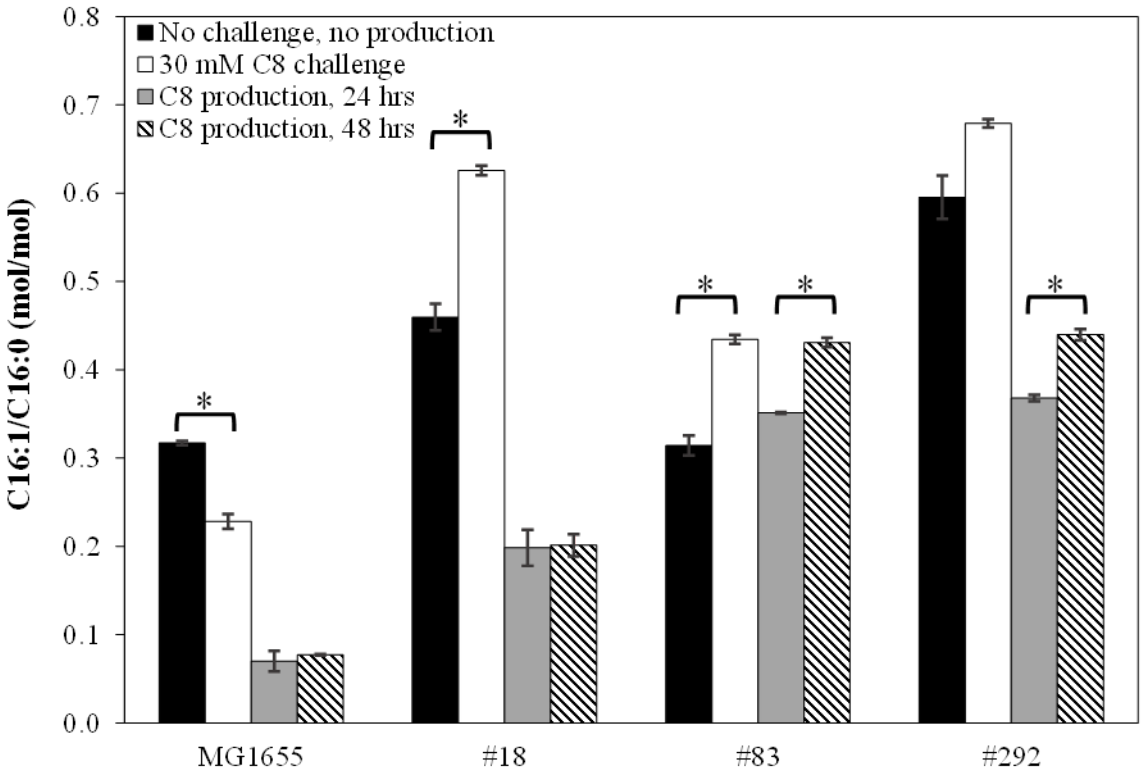

Figure 2. Increased growth during C8 challenge and increased C8 production by environmental isolates #83 and #292 are associated with an increase in C16:1 relative to C16:0 in the presence of C8. All strains were grown in MOPS minimal medium containing 2.0 wt% dextrose at 30 ºC with an initial pH 7.0. Values are the average of three biological replicates with error bars indicating one standard deviation.
Values are the average of three biological replicates with error bars indicating one standard deviation.
* indicates a significant difference ($p \leq 0.0006$), selected using the Bonferroni correction

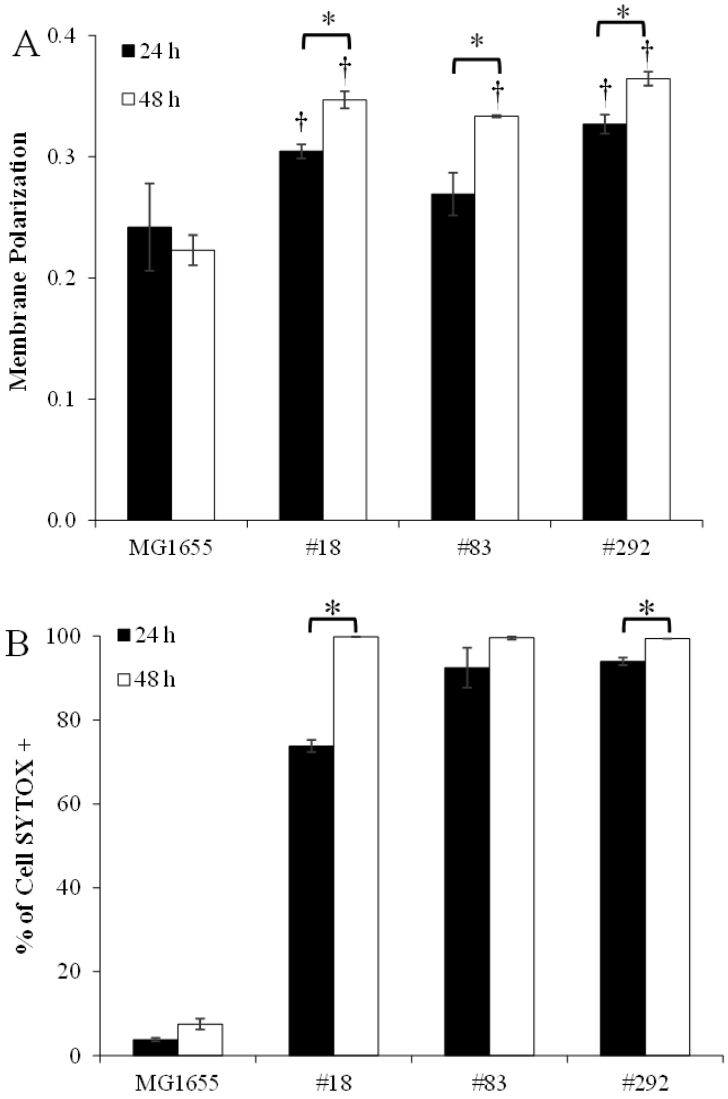

Figure 3. Membrane fluidity and integrity change during fatty acid production and differ for the environmental isolates relative to MG1655. All values are the average of three biological replicates with error bars indicating one standard deviation.
(A) Membrane polarization, which is inversely proportional to membrane fluidity, significantly increased in the environmental isolates during the course of C8 production.
(B) Membrane integrity, which is inversely proportional to SYTOX permeability, significantly increased during the course of fatty acid production in environmental isolates #18 and #292.
* indicates a significant difference ($p \leq 0.05$) between 24 hours and 48 hours for a single strain
† indicates a significant difference ($p \leq 0.05$) in the membrane polarization between MG1655 and environmental isolates, at 24 hours or 48 hours

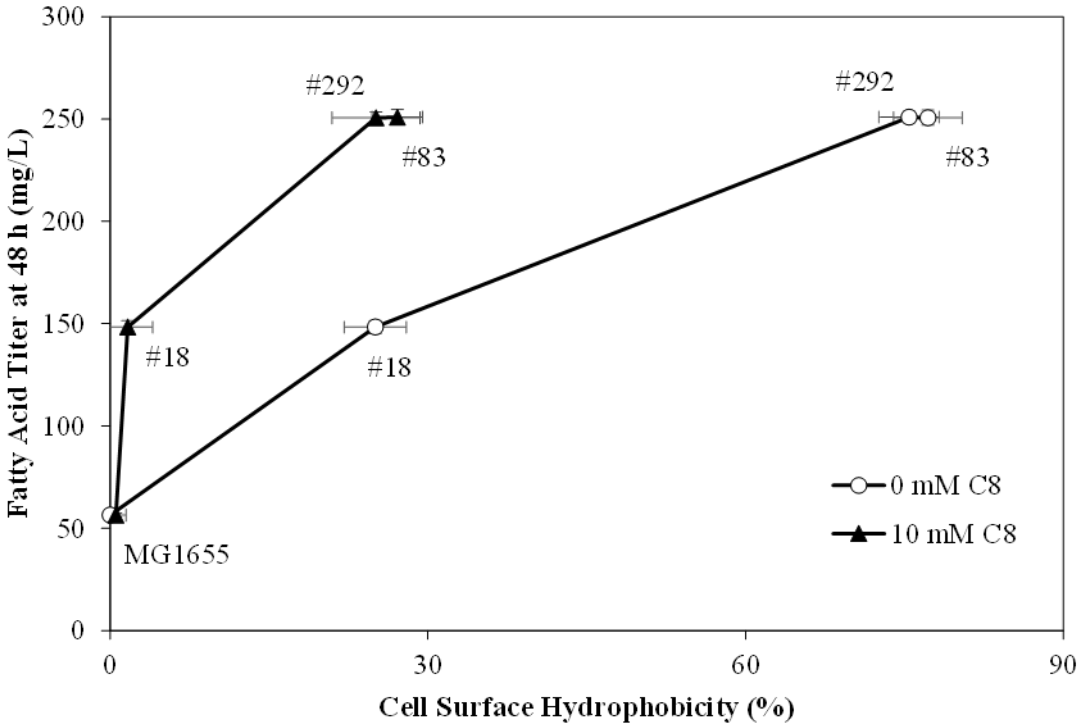

Figure 4. Increased fatty acid production is associated with higher cell surface hydrophobicity. Cell surface hydrophobicity was measured for mid-log cells growing in minimal medium with 0 mM or 10 mM C8 at 37 ºC, fatty acid titers are reported in Table 2. All values are the average of three biological replicates with error bars indicating one standard deviation.
All values are the average of three biological replicates with error bars indicating one standard deviation.

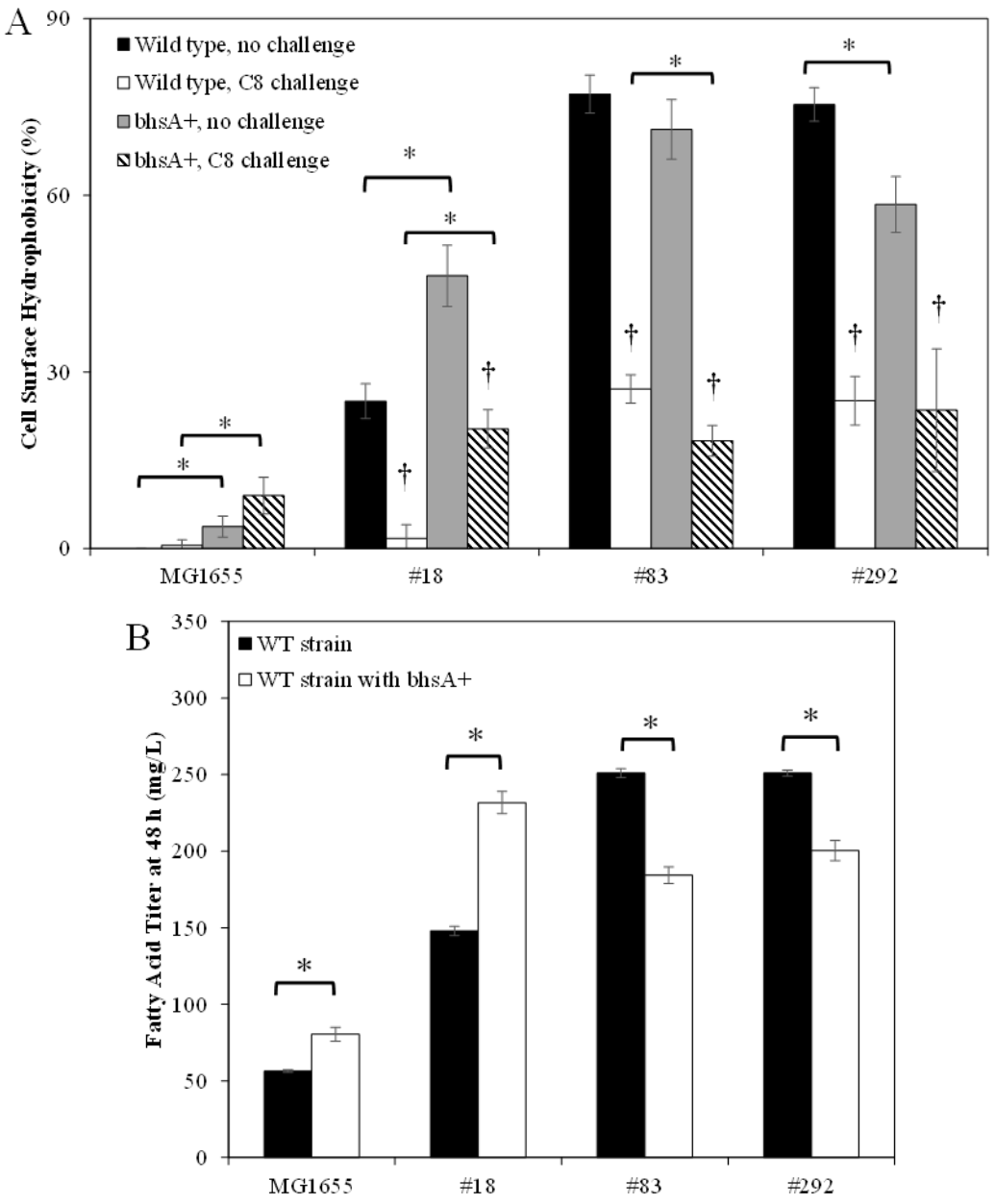

Figure 5. Expression tuning of *bhsA* alters cell surface hydrophobicity and fatty acid production. Values are the average of three biological replicates with error bars indicating one standard deviation.
(A) Cell surface hydrophobicity with and without exogenously provided C8. Cells were grown in MOPS minimal media at 37 ºC with and without 10 mM C8, with an initial pH of 7.0.
(B) Fatty acid production via expression of the *A. tetradius* thioesterase in MOPS minimal media at 30 ºC with an initial pH of 7.0.
† indicates a significant difference ($p \leq 0.05$) in the cell surface hydrophobicity between the two indicated conditions for the same strain
* indicates a significant difference ($p \leq 0.05$) in the cell surface hydrophobicity between wild type strain and the +*bhsA* strain for the same condition

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CHAPTER 7. SUMMARY AND FUTURE WORK

Summary

This work has focused on the identification of mechanisms that increase short-chain fatty acids (SCFAs) tolerance and production in *E. coli* with the goal of providing rational engineering strategies for improving robustness of microbial biocatalyst and increasing production of value-added biorenewable chemicals and fuels.

Although extensive studies have been done for improving fatty acid production of microbial biocatalysts, the SCFAs titers and yields still need further increase for achieving economic viability and compete with fossil fuels. One of the biggest challenge is SCFAs toxicity to microbes, which limits the cell growth and fermentation performance. It is known that membrane damage is one of the main mechanisms of the SCFAs toxicity to *E. coli* strains. In this study, we investigated the short-chain fatty acid-tolerant evolved strains and characterized a small set of environmental *E. coli* isolates with high short chain fatty acid production. All the efforts made were intended to understand the mechanisms of increased SCFAs tolerance and improved SCFAs production, which could be used as design strategies for rational engineering of microbial biocatalyst.

We applied genome-level reverse engineering to investigate the short-chain fatty acid-tolerance of the evolved strains. We identified four mutations and their chronological order of occurrence during adaptive evolution. Knowing the chronological order of the mutations in this study was very important, which helped us to have a better understanding about the evolved strains and provided a clear strategy to study each mutation and the synergistic interaction of them. Then we constructed 12 strains for further investigating how mutations contribute to the phenotypic changes of the evolved strain. In summary, the increased C8 tolerance phenotype was

observed after restoring the *waaG* and placing the mutant *rpoC* in the genome of parent strain. The mutant *rpoC* gene was the key mutation for improving fatty acid titer. The insertion sequence in the *waaG* gene present in the parent strain had a dramatic effect on the amount of extracellular polysaccharides. Likewise, by restoring the *waaG* gene the membrane leakage problem was solved. Interestingly, the *basS* and *basR* mutations were able to partially solve the membrane leakage problem when treating cells with 10 mM C8. The decreased membrane fluidity and increased cell surface hydrophobicity of evolved strain was recreated by the reconstructed strain which has the restored *waaG* gene, *rpoC* and *basR* mutations. The restored *waaG* gene and mutant *rpoC* gene both contributed to increased weight-average lipid length of the evolved strain. Thus, all mutations are important, especially the mutant *rpoC* resulted in increased C8 tolerance and high fatty acid titer. However, the *rpoC* and *basR* encode transcriptional regulators, which could influence on the expression level of many genes in *E. coli*. If we want to understand the mechanisms of *rpoC* and *basR* mutations, we have to utilize transcriptome analysis for further investigation.

In order to discover how the *rpoC* and *basR* mutations lead to phenotypic changes during fatty acid production, we did transcriptome analysis to identify differentially expressed genes between the evolved strain LAR1 and the parent strain ML115. Nineteen genes with differential transcript abundance likely caused by the *rpoC* mutation were selected, similarly twenty-one genes with differential transcript abundance likely caused by the *basR* mutation were chosen. Therefore, we constructed eighteen strains by knocking-out interesting genes or whole operons. Characterization of these reconstructed strains is in progress to further investigate the evolved strain.

We also characterized a small group of environmental *E. coli* isolates which had significant higher short-chain fatty acid production in minimal medium relative to the MG1655. In this study, we aimed to identify and prioritize membrane engineering strategies for improving fatty acid production in *E. coli*. We found differences in the cell membrane lipid composition, membrane fluidity, membrane integrity, and cell surface hydrophobicity between environmental isolates and MG1655. Consistent with the genome-level reverse engineering and previous publications, a decreased membrane fluidity was associated with an increased in fatty acid production. Surprisingly, cell surface hydrophobicity was also directly associated with fatty acid titer. For the cell membrane lipid composition, the C16:1/C16:0 ratio (mol/mol) was brought to our attention, since three environmental isolates all had a significantly higher ratio than the MG1655 strain during fatty acid production. Thus, we provided a new strategy for improving fatty acid production by rational altering the cell surface hydrophobicity. We also suggested that C16:1/C16:0 ratio could be a useful target for engineering the cell membrane composition.

To sum up, the first part of this work was to systematic investigate the evolved strain by genome-level reverse engineering and transcriptome analysis. Additionally, in order to deepen the understanding of cell membrane properties for improving short-chain fatty production, we investigated environmental *E. coli* isolates with high short-chain fatty acid titer. The findings obtained from this work could be used as rational engineering strategies for improving short-chain fatty acid tolerance and production of microbial biocatalyst.

Future Work

From the genome-level reverse engineering study, we noticed that the mutant *rpoC* gene resulted in an increased short-chain fatty acid tolerance and production. The evolved strain LAR1 had significant increased butanol tolerance relative to the parent strain ML115, as

previous studies reported. It would be interesting to know whether the *rpoC* mutation could also impact tolerance of other inhibitory compounds and environmental stressors, such us styrene, butanol, furfural, high temperature and low pH. Additionally, to find out whether the *rpoC* mutation could improve production of other desired toxic products, such as styrene, butanol and succinate might be an impactful strategy. The *rpoC* mutation may also contribute to increase tolerance and production of other inhibitory compounds in *E. coli*.

In the transcriptome analysis study, we focused on discover how the *rpoC* and *basR* mutations lead to the phenotypic changes of evolved strain LAR1. So, we selected the genes with differential transcript abundance between LAR1 and ML115 which were directly associated with the *rpoC* and *basR* genes. The transcriptome analysis results could provide more information for further investigation.

In the environmental isolates study, we altered the cell surface hydrophobicity by expressing the *bhsA* gene, in order to prove the directly association between cell surface hydrophobicity and fatty acid production titer. However, the direction and magnitude of the changes of cell surface hydrophobicity differed between strains. Thus, more reliable and stable strategies for altering cell surface hydrophobicity are needed for rational cell membrane engineering.